A STUDY OF 225 STRAINS OF *STAPHYLOCOCCUS* ISOLATED FROM THE MOUTH[1]

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The importance of *Staphylococcus* as an infective agent, especially in hospitals, needs no emphasis (McDermott, 1956; Editorial, 1956). Since the introduction of penicillin and its subsequent use on a large scale for a great variety of conditions, the attention of the medical profession and of microbiologists has been attracted by the ever-increasing proportion of antibiotic-resistant strains isolated from in-patients; this increase is, however, not so evident in the community at large (Rountree and Thomson, 1949; Rountree, Freeman and Barbour, 1954; Prissic, 1953), although a survey made in December, 1955 (Rountree and Rheuben, 1956), does indicate a small increase occurring over the previous 1½ years.

It seemed of interest to extend these observations on the distribution of *Staphylococcus* by studying 225 strains, half of which were isolated from a group of healthy children living as a semi-closed community in a children's home (Hopewood House, Bowral, New South Wales) and half from a group of healthy children of the general population (Metropolitan Sydney), especially in view of the fact that earlier investigations (unpublished) had indicated that almost all the subjects in the Bowral group harboured *Staphylococcus* in the mouth, insofar as these organisms could be isolated from the saliva on a high-salt agar medium (Chapman, 1945) enriched with 10 p.c. sheep blood.

MATERIALS AND METHODS.

Isolation of strains of Staphylococcus.

From the 83 children composing the Bowral colony samples of activated saliva were collected in the early morning before either food or drink was taken. The decision to use activated[3] saliva as the experimental material for the Bowral group was made because samples of saliva were being collected for other purposes and were thus readily available. Four to eight hours later saliva was distributed (by spreading 0·1 ml. with a sterile glass

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[3] Saliva obtained by stimulating the flow by chewing paraffin wax (m.p. 52° C.).

spreader) over the surface of nutrient agar containing 7·5 p.c. sodium chloride and 10 p.c. defibrinated sheep blood. The cultures were examined after incubation at 37° C. for 24 hours and 48 hours.

The plates were removed from the incubator and piled haphazardly on the bench and taken one by one for examination. From each plate as it was taken representative colonies showing various degrees of haemolytic activity and/or pigmentation were picked off for further examination. This process continued until between 100-120 colonies had been selected from 65 plates because it was considered that approximately 100 strains represented the smallest number which would yield significant results. The remaining plates (18 in number) were discarded.

From a large metropolitan school a group of 65 children corresponding as nearly as possible to the 65 children of the Bowral group in age- and sex-distribution was chosen. Saliva was obtained by rubbing dry sterile swabs, such as are used for making throat-swabbings, over the oral mucosa and teeth. It was not possible to obtain activated saliva because this would have seriously interfered with the procedure being followed by a team of workers engaged in a dental survey at the school.

From the 65 plates, sown by rubbing the swabs over the surface of the medium, 100-120 colonies were selected as before.

All strains destined for further examination, whether from the Bowral group or the Metropolitan Sydney group of children, were stored in Robertson's cooked meat medium at 5° C.

Examination of the strains.

From stock cultures in Robertson's cooked meat medium subcultures in broth were made. From these subcultures streak cultures on blood agar to test for purity and films stained by Gram's method to check the morphology of the organisms were prepared. Then agar slope cultures were prepared and after incubation for approximately 24 hours were used to make suspensions in distilled water. These suspensions were standardised (on the basis of opacity) to contain approximately 1,000 million micro-organisms per ml. One drop of suspension was then added to tubes of various test media, such as "sugar" media and nitrate broth. All test cultures were incubated at 37° C. except gelatin stab cultures, which were held at 16-18° C.; aerobic conditions were used throughout.

Results were recorded after incubation for 1, 2, 5 and 7 days except for gelatin cultures, which were examined after incubation for 7, 14, 21 and 28 days.

Bacteriophage-typing methods were applied to all strains producing coagulase for human plasma. Penicillin-sensitivity tests in duplicate on blood agar plates were made on all strains with the aid of penicillin-impregnated paper discs prepared in these laboratories and with Evans "Sentests" (0·5 unit of penicillin).

The tests used to characterise and to differentiate the species were the following:

(i) Colonial features, including pigment production on nutrient agar (24 or 48 hr. at 37° C. then 3 days at room temperature).

(ii) Morphology (including reaction to Gram's stain) in broth cultures.

(iii) Haemolysis: on sheep blood agar plates and on 7·5 p.c. salt blood agar plates.

(iv) Respiratory enzymes: production of catalase and methylene blue reductase; nitrate reduction.

(v) Proteolysis: gelatin-liquefaction (14 p.c. gelatin in nutrient broth), together with the production of indole, ammonia and hydrogen sulphide.

(vi) Fermentation: acid and gas production in 1 p.c. peptone water containing Andrade's indicator and 1 p.c. of glucose, sucrose, lactose, raffinose, inulin, salicin, glycerol or mannitol. When testing the ability of the organisms to attach starch 1 p.c. peptone water containing 0·1 p.c. of starch was used without indicator. Methyl red and Voges-Proskauer tests were also performed.

(vii) Litmus milk reactions: acid-production and clotting and reduction (decolorization) of the litmus.
(viii) Utilisation of inorganic nitrogen.
(ix) Coagulase-production.
(x) Penicillin-sensitivity.

Details of the performance of the various tests are set out below. All strains were tested three times before any conclusions were drawn as to their reactions, unless there was agreement between the first two series of tests.

Description of procedures.

Standardised suspensions of the strains were prepared in distilled water from 13-24-hour cultures on nutrient agar slopes. Test media (sugars, etc.) were inoculated with one drop of suspension delivered from a Pasteur pipette. At various periods after incubation the results were recorded.

Pigment-production. Nutrient agar plates were inoculated with a straight wire from standardised suspensions. The plates were incubated at 37° C. for 48 hours and then for 3 days at room temperature.

Haemolysis. 10 p.c. sheep blood agar plates were inoculated and incubated at 37° C. overnight. Observations were made also on 7·5 p.c. salt agar containing 10 p.c. sheep blood.

Catalase production. To nutrient agar plate cultures made from broth cultures 10 p.c. hydrogen peroxide was added and gas production observed. *E. coli* and the Oxford strain of *Staphylococcus* were used as controls.

Methylene blue reductase production. Small, narrow tubes of nutrient broth were inoculated with a large loopful of a broth culture and incubated for 24 hours at 37° C. Then from a Pasteur pipette 1 drop of 1 p.c. aqueous methylene blue was added; the contents of the tubes were well mixed and then covered with a layer of liquid paraffin. After incubation at 37° C. for 2 hours the tubes were observed for decolorization.

Nitrate reduction. 0·1 p.c. potassium nitrate broth was inoculated with one drop of standardised suspension and incubated at 37° C. for 5 days. Two methods were used to test for nitrate reduction: (a) On a white tile 2 drops of zinc chloride-potassium iodide-starch solution and 1 drop of hydrochloric acid (1/6) and one loopful of culture were mixed. A blue colour indicated the presence of nitrite (nitrate reduced); no colour indicated absence of nitrite (nitrate not reduced) (University of Melbourne, Practical Notes distributed by the School of Bacteriology); (b) 0·1 ml. sulphanilic acid solution was added to 5 ml. of culture, then α-naphthylamine solution was added drop-wise until a red colour appeared (usually 1-5 drops; with the control organism 10 drops fail to give a colour). A red colour indicated reduction of nitrate to nitrite while no change indicated the absence of nitrite (nitrate not reduced) (Zinsser's Textbook of Bacteriology, 1948).

Gelatin liquefaction. The medium used for this test was 14 p.c. gelatin in nutrient broth. For part of the first series of tests the gelatin was melted and one drop of standardised suspension added. Cultures were then incubated at 37° C. and before recording liquefaction were placed in a refrigerator for 1-2 hours. This method was found to be unsatisfactory. The method eventually adopted was to set the gelatin in the refrigerator and make stab cultures with a straight wire charged with organisms from nutrient agar slope cultures. The stab cultures were incubated at 16° C.

Indole production. Peptone water cultures were incubated for 5 days at 37° C.: then an equal volume of Ehrlich's reagent was added. A pink colour indicated the presence of indole, yellow its absence.

Ammonia production. Peptone water cultures were incubated for 5 days at 37° C. An equal volume of freshly prepared Nessler's reagent was then added. An orange-brown colour indicated the presence of ammonia, a pale yellow its absence.

Hydrogen sulphide production. Two drops of bacterial suspension were sown onto liver extract agar slopes. Sterile filter paper-strips, which had been soaked in 10 p.c. lead acetate solution and dried, were suspended above the slope. Hydrogen sulphide production was shown by darkening at the edge of the filter paper-strip.

Degradation of starch. The medium was inoculated with 1 drop of standardised suspension and incubated at 37° C. for 7 days. Gram's iodine was then added drop-wise until a blue colour persisted for some time. A record was kept of the number of drops of iodine required for control and culture. However, this test is not considered to be entirely reliable because, unless there is complete breakdown of all the starch present, the addition of iodine will produce a blue colour.

Methyl red test. Tubes containing 5 ml. of glucose phosphate peptone water were inoculated with a large loopful of a broth culture and incubated for 3 days at 30-37° C. Then 10 drops of methyl red solution in 60 p.c. alcohol were added. A red colour indicated a reaction, a yellow colour the absence of reaction.

Voges-Proskauer test. Tubes of glucose phosphate peptone water medium (as used for the methyl red test) were inoculated and incubated for 3 days at 30-37° C. 1 ml. potassium hydroxide was then added and the cultures incubated for a further period. After 4 hours a small amount of pink colour could be seen in the cultures. After 24 hours some cultures remained pale yellowish while others were a copper colour. The copper colour indicated a reaction and a pale yellowish colour its absence. *E. coli* (no reaction) and *Bact. aerogenes* (reaction) were used as controls.

Growth on synthetic medium. The medium used was that described by Bergey (1948, p. 236) containing ammonium phosphate as sole source of nitrogen.

Coagulase production. One drop of standardised suspension was added to 1 ml. of human plasma diluted 1/10 in nutrient broth. The results were observed after incubation at 37° C. for 4 hours and for 18-24 hours.

Sensitivity to penicillin. One loopful of standardised suspension was inoculated onto well-dried sheep blood agar plates. A paper disc containing 0·5 unit of penicillin was placed at the centre of the medium and organisms streaked from the edge of the disc to the edge of the plate (8 cultures per plate, one of which was the Oxford strain of *Staphylococcus*). For the Bowral group 'home-made' discs of filter paper were impregnated with 1 drop of penicillin solution to give 0·5 unit. For the Metropolitan Sydney group and for retesting any of the Bowral group (i.e., the resistant and some indeterminate strains) Evans "Sentest" discs (0·5 unit benzyl penicillin potassium salt) were used. Control plates with the Evans "Sentest" base were also observed.

'Phage-typing. Of all coagulase-producing strains approx. 16-hour cultures were plated on well-dried 1·2-1·5 p.c. nutrient agar plates and allowed to dry. 'Phages were "spotted" on with a fine Pasteur pipette. After the 'phage spots had dried the plates were incubated at 30° C. overnight.

The pattern of the lysis was recorded as: confluent lysis, ++++; 50-100 plaques, ++; less than 50 plaques, +; no plaque, −. The 'phages used were:

Group I: 29, 44, 52, 52A (var. now called 80), 79.
Group II: 3A, 3B, 3C, 55.
Group III: 6, 7, 31B, 42E, 47, 47A, 47C, 47D, 53, 54, 70, 73, 75, 77.
Group IV: 42D.

('Phage-typing was carried out under the supervision of Dr. Phyllis Rountree.)

RESULTS.

Classification of strains on the basis of biological characters.

Table 1 shows the number of strains which reacted when subjected to the various tests. Where satisfactory data were not available for all strains in the series, the results are expressed in terms of the number of strains for which such data are available, e.g., for the methyl red test 209/217 not 209/225.

TABLE 1.

Biological characteristics of 225 strains.

Test or Characteristic	No. strains which reacted
Pigment, golden	62
yellow	34
cream	43
white	93
Morphology conformable to recognised description	225
Haemolysis on sheep blood agar	146
Haemolysis on high-salt sheep-blood agar	193
Catalase	*223/224
Methylene blue reductase	223/223
Nitrate reduction	154
Proteolysis: Gelatin	151
Indole	0
Ammonia	133
Hydrogen sulphide	201
Fermentation: Glucose	225
Sucrose	225
Lactose	211
Raffinose	2
Starch	0
Inulin	0
Salicin	3
Glycerol	205
Mannitol	137
Methyl red test	209/217
Voges-Proskauer test	116/224
Litmus milk: Acid and clot	111
Acid but no clot	45
Alkaline	57
No change	12
Growth on synthetic medium	0
Coagulase	79
Penicillin-sensitivity	193

* The records for one strain were deficient in relation to catalase-production so that only 224 strains are shown as tested for catalase. One of these 224 strains failed to give evidence of catalase-production, but is included as a *Staphylococcus* because its other properties were in conformity with the accepted definition.

On the basis of the descriptions (Table 2) set out in Bergey's Manual of Determinative Bacteriology (1948), we have classified the strains as follows (Table 3).

TABLE 2.

Physiological reactions used to characterise the various species.

(After Bergey, except for coagulase-production.)†

Species, variety or strain	Pigment	Coagulase*	Glucose	Lactose	Sucrose	Raffinose	Mannitol	Starch	Salicin	Inulin	Glycerol	Litmus milk	Gelatin	Indole	Nitrate	Ammonia	Synthetic medium	Hydrogen sulphide
pyogenes, var. *aureus*	yellow to orange	+	+	+	+	−	+	−	−	−	+	AC	+	−	+	+	−	(+)
pyogenes, var. *albus*	white	+	+	+	+	−	+	−	−	−	+	AC	+	−	+	+	−	+
epidermidis	white	−	+	+	+	−	−	0	−	−	0	A	+*	−	+	0	∓	0
aurantiacus	yellow to orange	0	(+)	(+)	(+)	−	(+)	−	−	−	0	(A)	−	−	±	+	−	0
candidus	white	0	+	+	+	0	0	−	0	0	+	(A)	−	−	−	+	−	0
flavus	pale yellow	0	±	±	∓	0	∓	−	0	0	∓	(A) C (R)	+	−	−	+	−	0
citreus	yellow	0	+	+	+	+	+	−	+	+	+	AC*	+*	−	+	+	−	0
alkaline milk	white	−	+	−	+	−	+	−	−	−	+	alk	+	−	+	+?	−	+
	yellow	−	+	+	+	−	−	−	−	−	+	alk	−	−	+	+?	−	+
lactose non-fermenting	cream	−	+	−	+	−	−	−	−	−	+	alk CR	+	−	+	+?	0	+
lactose non-fermenting: gelatin-non-liquefying	yellow	−	+	−	+	−	+	−	−	−	+	alk	−	−	+?	+	0	+

† For purposes of our study we have modified the characters attributed by Bergey to *Staphylococcus pyogenes* by including coagulase-production as an essential quality.

+ = unequivocal reaction; (+) = always slight; ± = generally reacts; ∓ = generally does not react; * = slow reaction; 0 = no data given by Bergey; A = acid; C = clot; R = removal of colour.

TABLE 3.

Classification of the 225 strains of Staphylococcus (Micrococcus).

(Bergey)

Species*	No. strains	p.c. strains
pyogenes—typical	21	79	35·1
atypical	58		
epidermidis—typical	3	59	26·4
atypical	56		
aurantiacus—typical	2	33	14·7
atypical	31		
candidus—typical	1	33	14·7
atypical	32		
flavus—typical	2	19	8·4
atypical	17		
citreus—typical	0	2	0·9
atypical	2		

* Note: Species are designated 'atypical' if they failed to conform to the descriptions given in Bergey's Manual.

The number of coagulase-producing strains was 79 (35·1 p.c.) and all these have been classified as *Staphylococcus pyogenes*. All coagulase-producing strains fermented glucose, sucrose and lactose and all, except one strain accidentally omitted, reacted to the methyl red test.

On the basis of the descriptions given by Shaw, Stitt and Cowan (1951) (Table 4) the strains can be classified as set out in Table 5.

TABLE 4.

Physiological reactions used to characterise the various species.

(after Shaw, Stitt and Cowan)

Species	Group	Pigment	Coagulase	Glucose	Acetoin (Voges-Proskauer)	Nitrate redn.	Methylene blue	Gelatin	Urease	Milk	Arrangement of cells
aureus	I	golden or cream, white often	+*	A*	+	+	+	+	+	A	
saprophyticus	II	white	−*	A*	+*	+	+	+	+	A	
lactis	III	not pink	−*	A*	−*	+	+	+ or −	+ or −		tetrads or packets

* essential characteristic.

TABLE 5.

Classification of 224* *strains of* Staphylococcus

(Shaw, Stitt and Cowan)

Species†	No. strains	p.c. strains
Group I—*aureus*—typical	33	34·8
atypical	45 } 78	
Group II—*saprophyticus*—typical	48	33·0
atypical	26 } 74	
Group III—*lactis*—typical	28	32·1
atypical	44 } 72	

* One strain was omitted from this table because no data were available for methylene blue reductase and the Voges-Proskauer test.

† Note: Species are designated 'atypical' if they failed to conform to the descriptions given by Shaw, Stitt and Cowan.

The relation between these two classifications is discussed below.

Correlation between coagulase-production and various properties listed by Bergey (1948).

Accepting coagulase-production as the paramount indication of pathogenicity (Jackson *et al.*, 1955, argue strongly in favour of determining the α-haemolysin titre as a test for pathogenicity in man) this property has been correlated with various other properties of the organisms and the results are set out in Table 6.

TABLE 6.

Correlation between coagulase-production and various other properties of the 225 strains.

	Number of strains		
	+ve	−ve	r*
Haemolysis on sheep blood agar:			
Coagulase-producing	75	4	0·46
Coagulase non-producing	71	75	
Haemolysis on high-salt sheep blood agar:			
Coagulase-producing	74	5	0·17
Coagulase non-producing	119	27	
Nitrate reduction:			
Coagulase-producing	64	15	0·20
Coagulase non-producing	90	56	
Gelatin liquefaction:			
Coagulase-producing	78	1	0·50
Coagulase non-producing	73	73	
Ammonia production:			
Coagulase-producing	73	6	0·50
Coagulase non-producing	60	86	
Hydrogen sulphide production:			
Coagulase-producing	68	11	0·08
Coagulase non-producing	133	13	
Glycerol fermentation:			
Coagulase-producing	69	10	0·10
Coagulase non-producing	136	10	
Mannitol fermentation:			
Coagulase-producing	78	1	0·56
Coagulase non-producing	60	86	

* $r = \sqrt{\frac{\chi^2}{N}}$, where N = total number of observations made.

Rountree (1956) noted the relative scarcity of information concerning the antibiotic resistance of coagulase non-producing strains and reports that in one series 50 of 444 such strains were penicillin-resistant and in other series approximately 17 p.c. of 110 strains.

Of 146 such strains in our series there were only 11·6 p.c. penicillin-resistant strains.

Table 7 shows the distribution of all the 225 strains according to coagulase-production and penicillin-sensitivity.

TABLE 7.

Correlation between coagulase-production and penicillin-sensitivity.

	Penicillin-sensitive	Penicillin-resistant	r‡
Coagulase-producing	64 (28·4 p.c.)	15* (6·7 p.c.)	0·10
Coagulase non-producing	129 (57·3 p.c.)	17† (8·0 p.c.)	

* 15/79 (19 p.c.) of the coagulase-producing strains were resistant.
† 17/146 (11·6 p.c.) of the coagulase non-producing strains were resistant.
‡ See footnote Table 6.

The correlation between haemolysis on high-salt sheep-blood agar and that on sheep-blood agar is shown in Table 8.

TABLE 8.

Correlation between haemolysis on high-salt sheep-blood agar and that on sheep-blood agar.

	Haemolysis on high-salt sheep-blood agar +ve	Haemolysis on high-salt sheep-blood agar −ve	r*
Haemolysis on sheep-blood agar: +ve	144	2	0·49
Haemolysis on sheep-blood agar: −ve	50	29	

* See footnote Table 6.

Classification of coagulase-producing strains according to 'phage patterns.

Table 9 shows the distribution of 'phage patterns within the groups.

TABLE 9.

Classification of coagulase-producing strains according to 'phage group.

'Phage group	'Phage pattern	No. strains	Total No. strains in group
I	52A (var. 80)	3	10
	52	1	
	29	3	
	29/52	2	
	52/52A	1	
II	3C/55	5	12
	3B/3C/55	5	
	55	1	
	3A/3B	1	
III	42E/6/7/54/73	1	10
	6/7/47/53/54/75/31B/47C	2	
	6/7/47/53/54/75/31B	2	
	7/31B/47D	1	
	6/7/47/53/54/75/47C	1	
	6/7/47/53/54/73/75	1	
	6 (weak)	1	
	7 (weak)	1	
IV	42D	4	4
Non-classifiable	42D/75	1	13
	42D/44	2	
	42D/6/7/54/73/75	1	
	52/52A/6/79/47C	1	
	42D/29/52/52A	2	
	52/52A/5/47C	1	
	42D/42E/6/54/73	1	
	29/77	1	
	42D/29/44/52/52A/42E/6/7/47/54/73/75	1	
	42D/29/44/52/52A/42E/6/7/47/53/54/73/75	1	
	3A/6/73/75	1	
Non-typable			30
Total			79

Classification of coagulase-producing strains according to their 'phage reactions and their penicillin-sensitivity.

Table 10 shows for coagulase-producing strains the distribution of the 'phage groups according to the strains' reaction to penicillin.

TABLE 10.

Distribution of coagulase-producing strains according to their reactions towards penicillin and the different 'phage groups.

Coagulase-producing strains	Number of strains						
	'Phage groups					Non-typable	Total
	I	II	III	IV	Non-classifiable*		
Penicillin-sensitive	10	12	4	3	13	22	64
Penicillin-resistant	0	0	6	1	0	8	15
Total	10	12	10	4	13	30	79

* The non-classifiable strains are as follows:—
1 strain reacted to 'phages of groups II and III,
3 strains reacted to 'phages of groups III and IV,
4 strains reacted to 'phages of groups I and IV,
5 strains reacted to 'phages of groups I and III.

Distribution of 'phage groups according to location of the strains.

Table 11 shows for both penicillin-sensitive and penicillin-resistant strains the distribution of strains from various locations of the body according to the 'phage group. Apart from our own series from mouths these strains were isolated from normal noses, normal throats and various infections.

Table 12 shows similar information for a series of strains isolated from the staff of a large hospital and compares it with our own series of strains isolated during the same year.

TABLE 11.

Distribution (numbers and percentages) of the 'phage groups according to source of the strains and according to their reaction towards penicillin.

Source	No. strains	'Phage groups				
		I	II	III	IV	Non-classifiable (incl. non-typable)
				Penicillin-sensitive		
*Normal noses	387	19·2 p.c. (74)	19·9 p.c. (77)	26·9 p.c. (104)	—	33·9 p.c. (131)
*Normal throats	102	17·7 p.c. (18)	19·6 p.c. (20)	28·4 p.c. (29)	—	34·3 p.c. (35)
Normal mouths	64	15·6 p.c. (10)	19·0 p.c. (12)	6·2 p.c. (4)	4·7 p.c. (3)	54·7 p.c. (35)
*Infections (various)	880	21·2 p.c. (187)	27·7 p.c. (244)	23·1 p.c. (203)	—	28·0 p.c. (246)
				Penicillin-resistant		
*Normal noses	273	2·5 p.c. (7)	0·5 p.c. (1)	78·7 p.c. (216)	—	18·3 p.c. (50)
*Normal throats	107	8·4 p.c. (9)	0·0 p.c. (0)	66·4 p.c. (71)	—	25·2 p.c. (27)
Normal mouths	15	0·0 p.c. (0)	0·0 p.c. (0)	40·0 p.c. (6)	6·7 p.c. (1)	53·3 p.c. (8)
*Infections (various)	931	7·2 p.c. (67)	1·5 p.c. (14)	75·8 p.c. (706)	—	15·6 p.c. (145)

* Adapted from Rountree (1953)

TABLE 12.

Distribution (numbers and percentages) of the 'phage groups according to source of the strains and according to their reactions towards penicillin.*

Source	No. strains	'Phage groups				
		I	II	III	IV	Non-classifiable (incl. non-typable)
		Penicillin-sensitive				
†Normal noses	30	23·3 p.c. (7)	20·0 p.c. (6)	30·0 p.c. (9)	0·0 p.c. (0)	26·6 p.c. (8)
Normal mouths	64	15·6 p.c. (10)	18·8 p.c. (12)	6·2 p.c. (4)	4·7 p.c. (3)	54·7 p.c. (35)
†Lesions	38	29·0 p.c. (11)	29·0 p.c. (11)	23·7 p.c. (9)	0·0 p.c. (0)	18·5 p.c. (7)
		Penicillin-resistant				
†Normal noses	112	13·4 p.c. (15)	0·0 p.c. (0)	76·0 p.c. (85)	0·9 p.c. (1)	9·8 p.c. (11)
Normal mouths	15	0·0 p.c. (0)	0·0 p.c. (0)	40·0 p.c. (6)	6·7 p.c. (1)	53·3 p.c. (8)
†Lesions	80	55·0 p.c. (44)	2·5 p.c. (2)	33·8 p.c. (27)	1·2 p.c. (1)	7·5 p.c. (6)

* All strains listed in the Table were isolated during 1954.
† After Rountree and Freeman (1955).

As other workers have shown (Wise *et al.*, 1954; Knight *et al.*, 1954) the penicillin-resistant strains fall mainly into group III, although (Table 12) this is not so for "lesion" strains isolated during the year 1954 from hospital personnel: most strains fell into group I.

Dissection of variant reactions of species as determined according to Bergey's Classification.

From Table 3 it is clear that many strains have had to be classified as 'atypical' because they failed to react appropriately upon one or more of the various substrates. Table 13 summarises the atypical reactions of the 225 strains according to species.

TABLE 13.

The distribution of variant reactions among the 225 strains according to species.

Species and no. strains in each		Test									
		Gelatin	Lactose	Glycerol	Ammonia	Nitrate redn.	Hydrogen sulphide	Litmus milk	Mannitol	Salicin	Inulin
pyogenes	79	1	0	9	6	15	12	32	1	2	0
epidermidis	59	4	1	0	0	4	0	54	3	0	0
aurantiacus	33	0	8	0	20	0	0	28	12	0	0
candidus	33	0	0	0	17	1	0	31	26	0	0
flavus	19	2	0	0	6	11	1	8	1	0	0
citreus	2	1	0	0	0	0	0	1	0	1	2
Total	225	8	9	9	49	31	13	154	43	3	2

DISCUSSION.

The results of this study provide data upon the biological reactions of 225 oral strains of *Staphylococcus*. The majority of strains, i.e. 85 p.c. or more, are Gram positive cocci occurring on solid media in irregular bunches, but in liquid media as single cells, pairs, small groups (about 6 cells) or arranged in short chains (rarely more than five cells); appear to produce haemolysis on a high-salt sheep-blood agar; produce catalase, hydrogen sulphide and methylene blue reductase; ferment glucose, lactose, sucrose and glycerol; react to the methyl red test and are sensitive to penicillin; fail to produce indole, to ferment raffinose, starch, salicin, inulin and to grow on a synthetic medium containing ammonium phosphate as the sole source of nitrogen.

The results support the generally accepted view that there are considerable variations in the biological reactions of strains of *Staphylococcus* but inherent in the statements given above concerning them is a description[4] of the strains of *Staphylococcus* which we found in the mouth of children aged 4 to 11 years.

On the criteria set out in Bergey's Manual of Determinative Bacteriology (1948) altogether six species were recognised, all of which grew under aerobic conditions: *pyogenes, epidermidis, aurantiacus, candidus, flavus* and *citreus*. These were each divisible into typical and atypical groups, according as they did or did not conform to the descriptions given in Table 2, which is based on the properties set out in the Manual.

It seems clear that a large proportion of the tests listed in Table 13 are much too unreliable to be of much value in classification. For the important pathogen, *Staphylococcus pyogenes*, the following reactions were aberrant in more than 10 p.c. of strains: glycerol-fermentation, nitrate-reduction, hydrogen sulphide-production, acidification of litmus milk.

On the basis of criteria suggested by Shaw, Stitt and Cowan, three species were recognised: *aureus, saprophyticus* and *lactis*. These were each divisible into typical and atypical according as they did or did not conform to the description given in Table 4, which is based on the properties listed by Shaw, Stitt and Cowan (1951).

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[4] Spheroidal cells, staining Gram-positive; when grown on solid media arranged in clusters, but when grown on liquid medium arranged singly or in pairs, small groups of 4-8 and short chains of 3-6; generally fermenting glucose, lactose, sucrose and glycerol and producing hydrogen sulphide; generally failing to attack raffinose, starch, salicin and inulin; always producing methylene blue reductase; generally giving the methyl red reaction; always failing to use inorganic nitrate as the sole source of nitrogen and not producing indole; generally showing haemolytic activity on sheep-blood agar containing 7·5 p.c. sodium chloride. The following reactions or properties are variable: haemolysis on sheep-blood agar, production of coagulase, production of ammonia, reduction of nitrate, liquefaction of gelatin, fermentation of mannitol, reaction to the Voges-Proskauer test; pigmentation of colonies on nutrient agar and blood agar; reactions in litmus milk.

Although an analysis of variant reactions (according to Cowan's criteria) has not been made, it is clear that variability in biological characteristics remains a major difficulty in devising a satisfactory system of classification (see Table 14 where the percentage of typical and atypical strains (according to Bergey's and Cowan's criteria) are compared).

TABLE 14.

Comparison of the proportions of typical and atypical strains according to the criteria of Bergey and of Shaw, Stitt and Cowan respectively.

Strains	Bergey		Shaw, Stitt and Cowan	
	Number	p.c.	Number	p.c.
typical	29	12·9	109	48·7
atypical	196	87·1	115	51·3
Total	225	100	224*	100

* See note Tables 3 and 5.

Correlation between the two systems of classification.

Omitting reference to minor departures from type characteristics the strains were allocated to the relevant groups under the two classifications in an attempt to achieve a correlation. Table 15 shows an absolute correlation between *Staphylococcus* (*Micrococcus*) *pyogenes* (Bergey) and *Staphylococcus aureus* (Shaw, Stitt and Cowan). This is understandable insofar as we have made both to depend upon the production of coagulase as the criterion of pathogenicity.[5] A fairly satisfactory correlation exists between *Staphylococcus* (*Micrococcus*) *epidermidis* (Bergey) and *Staphylococcus saprophyticus* (Shaw, Stitt and Cowan)—51 of 59 strains. A high degree of correlation exists between *Staphylococcus* (*Micrococcus*) *candidus* (Bergey) and *Staphylococcus lactis* (Shaw, Stitt and Cowan)—31 of 33 strains. The remaining 54 strains which according to Bergey's criteria would fall into the species *aurantiacus, flavus* and *citreus* were distributed between *Staphylococcus saprophyticus* and *Staphylococcus lactis* (Shaw, Stitt and Cowan)—21 in *saprophyticus* and 33 in *lactis*.

Greer (1956) in a study of 200 strains of *Micrococcus* from various skin lesions suggests still another basis for classification, whereby *M. pyogenes, M. citreus* and *M. aurantiacus* are merged into one species, and claims that such a scheme is compatible with previous reports by other investigators. No attempt has been made to classify our strains according to Greer's criteria.

[5] See footnote to Table 2.

TABLE 15.

Correlation between the two systems of classification.

Classification of Bergey	Classification of Shaw, Stitt and Cowan		
	I *aureus*	II *saprophyticus*	III *lactis*
pyogenes	78*	0	0
epidermidis	0	51	8
aurantiacus	0	13	20
candidus	0	2	31
flavus	0	8	11
citreus	0	0	2

* One strain omitted: see Table 5.

Pathogenicity.

In this connection it is of interest to compare coagulase-production, mannitol-fermentation and haemolysis on sheep-blood agar as criteria of pathogenicity. Strains which produce coagulase were accepted as pathogenic and classified by us as *pyogenes*.

If we limit ourselves to the strains classified as *pyogenes* we find that only one failed to ferment mannitol. Accepting coagulase-production as indicative of pathogenicity we have 79/225 pathogenic strains. As stated above, one of these 79 failed to ferment mannitol. Hence, if we accept mannitol-fermentation as indicative of pathogenicity we fail to detect 1 of the 79. Thus, we would have an error of omission of 1·27 p.c. (1/79). Again, accepting mannitol-fermentation as indicative of pathogenicity, we have in all 137/225 pathogenic strains, that is, we have detected falsely an excess of 137 minus 79, i.e. an excess of 58 strains, which are really non-pathogenic (coagulase non-producing). The real number of non-pathogenic strains according to the coagulase test is 225 minus 79, i.e., 146, therefore, reliance on fermentation of mannitol could lead to an error of commission of 39·7 p.c. (58/146).

Because haemolysis on blood agar is generally the first evidence obtained in the laboratory of possible pathogenicity the correlation of this property with coagulase-production was determined. Only four strains classified as pathogenic *pyogenes* failed to show haemolysis (Table 6). Nonetheless, it is somewhat disconcerting, at least to the clinical bacteriologist, to find 4/64 pathogenic strains failing to show haemolysis on blood agar plates.

'Phage typing of the coagulase-producing strains did not reveal a preponderance of any particular strain (Table 10). There is, however, a rather larger proportion of non-typable and Group 4 strains in our series than is usual in a collection of strains isolated from the nose, throat and lesions.

SUMMARY.

Two hundred and twenty-five strains of *Staphylococcus* isolated from the mouths of 130 healthy children were classified (a) on the basis of a series of physiological reactions as set out in Bergey's Manual, and (b) according to the scheme suggested by Shaw, Stitt and Cowan.

The results of the study confirm the generally acknowledged variability of this organism's biological characteristics and the difficulties in determining satisfactory criteria for the establishment of species.

Of the 225 strains studied, 79 were coagulase-producers and were therefore regarded as pathogenic to man.

Among the 79 pathogenic strains 15 were penicillin-resistant.

'Phage typing failed to demonstrate any predominant strain.

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THE EFFECTS OF PROLONGED SUB-TOXIC LITHIUM INGESTION ON PREGNANCY IN RATS

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The beneficial effect of lithium medication on mania and other states of psychomotor excitement has been reported and confirmed by a number of independent workers (Cade, 1949; Noack *et al.*, 1951; Glesinger, 1954; Schou *et al.*, 1954; Plichet, 1954). Control of the plasma lithium levels (Trautner *et al.*, 1955) allows a safe management of the medication; the symptoms and treatment of accidental intoxications have been described by Coats *et al.*, (1957). In consequence, many patients can be released from hospital to continue the medication under the supervision of their physicians.

No untoward effects of prolonged lithium ingestion on health or functional efficiency have so far been observed even in patients who, with only occasional interruptions, continued the medication for periods of 4 to 5 years. The question of medication, however, in a case of pregnancy in a lithium-treated patient, is still open. Should one discontinue the medication and risk a recurrence of mania? Or, if lithium ingestion is continued, does it involve possible damage to the mother or the foetus?

Lithium salts are known to affect cleavage and therefore survival of the invertebrate (Needham, 1942) and amphibian egg (Lallier, 1953). MacLeod *et al.* (1949) and Dollander and Laurent (1952) reported a reduction in metabolic rate and motility of rat spermatozoa brought into a lithium-containing medium. Bass (1951) observed a reduction in the number of the viable litter following subcutaneous injection of lithium chloride in mice on the 2nd to 7th day of pregnancy. The interpretation of these observations with regard to the safety of lithium medication in man is difficult for the following reasons: The oral or parenteral administration of a quantity of lithium chloride causes temporarily high, possibly toxic plasma lithium concentrations (Trautner *et al.*, 1955), which may affect the embryonal tissue even if the adult animal suffers no more than transient nausea. Repeated injection or other administration connected with actual handling, on the other hand, constitutes by itself a stress which could

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possibly affect the somatic and behavioural responses of the animal. Furthermore, at the start of lithium medication there is a transient disturbance of ionic homoeostasis and possibly other somatic functions which may affect a pregnancy existing or occurring during that period.

For these reasons it was decided to administer lithium chloride in the drinking water of female rats so that its ingestion is distributed over the whole day; and to start observations with regard to pregnancy only after the animals could be regarded as being well adapted to, and well stabilized on, their lithium-containing diet.

In the present paper the effects of maintained lithium ingestion in female rats before and during pregnancy will be presented. The observations were directed (a) to establish a lithium tolerance limit, that is a concentration of lithium chloride in the drinking water which is indefinitely compatible with full health and normal behaviour of the animals and comparable to the lithium levels maintained in man; (b) to assess the extent of the initial disturbance caused by the introduction of an ultimately well tolerated lithium dose, to exclude the effects of an acute stress on somatic function, and (c) to record the frequency and fate of any pregnancies occurring during the period of regular steady lithium ingestion, especially the health and behaviour of the mother during pregnancy, birth and lactation, and the number, viability and progress of the young.

Materials and Methods.

Several strains of laboratory rats were used in preliminary experiments. These strains differed slightly in degree only in their tolerance or responses to lithium. The animals used for the series recorded were albino Wistar rats, laboratory bred for a number of years. The range of weight for the adult animals was 140 to 180 gm. for the female and 200 to 270 gm. for the male. The oestrus cycle was 4 days; the period of gestation 22 to 24 days.

To avoid the effects of too much handling in the actual test series, water and food intakes, and weight changes were measured independently in parallel batches of 6 to 8 animals.

The water intake of the animals was measured by using graduated bottles dripping into a drinking trough to prevent water losses due to temperature-induced changes in the air content of the bottles. The daily intake was 10 to 12 gm. per 100 gm. weight. The food intake of the animals was difficult to measure. Whether it was offered pressed into cubes, or finely granulated, a certain amount of waste could not be avoided. It was therefore assumed that the difference in weight of food boxes, too small to allow the animals to sleep in them, gave at least a relative measure of the intake. This was about 7 gm. per 100 gm. weight per day. Lithium was offered as the chloride, dissolved in the drinking water in various concentrations. The use of lithium citrate showed within the range of well tolerated doses no advantages or differences. No other salts were tried.

The determinations of plasma lithium, sodium and potassium levels were made on blood specimens obtained by cardiac puncture of the ether-anaesthetized animals. The cations were estimated by a Beckman model DU flame spectrophotometer.

For mating experiments, test and control animals were put at the same time in identical boxes and kept under the same conditions in the same room. In the evening two males were put into a box containing six females, and the drinking water was removed from all boxes for the night. In the morning the males were removed; in the evening the same females were put with two other males overnight. Vaginal smears were made every morning, and service was assumed if spermatozoa were found in the smear. The serviced females

were separated; the day after service was counted as day 1 of the pregnancy. The procedure was found to be reliable only if the animals were undisturbed for less than about eight hours. After that period the incidence of service could not necessarily be confirmed in the smear. In all series recorded in detail, service was ascertained by examination of the smear.

Laparotomies were performed on the ether-anaesthetized animals under clean conditions by a midline abdominal incision. Corpora lutea, implantation sites and foetuses were counted. After inspection the abdominal muscles and skin were sutured separately with cotton and each rat was placed in an individual nursing box. Recovery from the anaesthetic was complete within half an hour and the wound healed quickly with very little infection. The operation had very little effect on the course of the pregnancy. In only a few of almost one hundred was the whole litter still-born.

RESULTS.

Lithium tolerance.

Adult rats put on drinking water containing 50 mEq of lithium chloride per litre appeared to be unchanged in aspect and behaviour for 3 to 5 days. Then they became progressively drowsy and asocial. They were difficult to rouse; when roused, their gait was staggering and hesitant and they rested again as soon as possible. When resting they showed fine muscular tremor and trembling; usually they sat for long periods by themselves anywhere in the box. A few days later they became unresponsive and were stuporous, with dull eyes and slightly erected, untidy and unkempt fur. They were visibly ill and emaciated. Death occurred within 2 to 3 weeks. There was no noticeable apprehension or irritability during the early stages, and there was neither diarrhoea nor convulsions at any stage.

Effects of the lithium intake could be observed before the behaviour of the animals changed. The food and water intake fell to about 50 p.c. of the normal within a few days. The weight began to drop from the 3rd and 4th days by 2 to 3 gm. per 100 gm. per day. Pronounced dehydration with indrawn belly and wrinkled abdominal skin were apparent from the 8th to 10th day. The animals died uniformly when their weight had dropped by 40 to 45 p.c.

The plasma lithium levels increased steadily. They were about 3 mEq/l when the first changes in behaviour became apparent, reached about 7 mEq/l during the second week and exceeded 8 mEq/l just before death. Except for gross dehydration and fat loss, the inspection of the dead animals revealed no macroscopic damage. It appeared that no early irreversible change was caused, since, up to a weight loss of about 30 p.c., that is up to within a few days of certain death, all animals recovered without noticeable after effects within one week, and regained their previous weight within two weeks, if they were put on pure, lithium-free drinking water.

No direct observations could be made as to whether the first lithium effect consisted in a gross anorexia followed by reduced fluid intake. However, toxic animals avidly ate the dead animals without showing any signs of graver intoxication. This may indicate that their appetite was not impaired and that they took to food with a lower lithium concentration than that present in the drinking

water (10-12 mEq/1 in the dead animals compared with 50 mEq/1 in the drinking water).

With 30 mEq/1 of lithium chloride in their drinking water some of the animals lost weight progressively, became ill and died within 3 to 4 weeks with the same symptoms as those on 50 mEq/1. But others in the same batch and in the same box reached and maintained, after an initial weight loss of 10 to 20

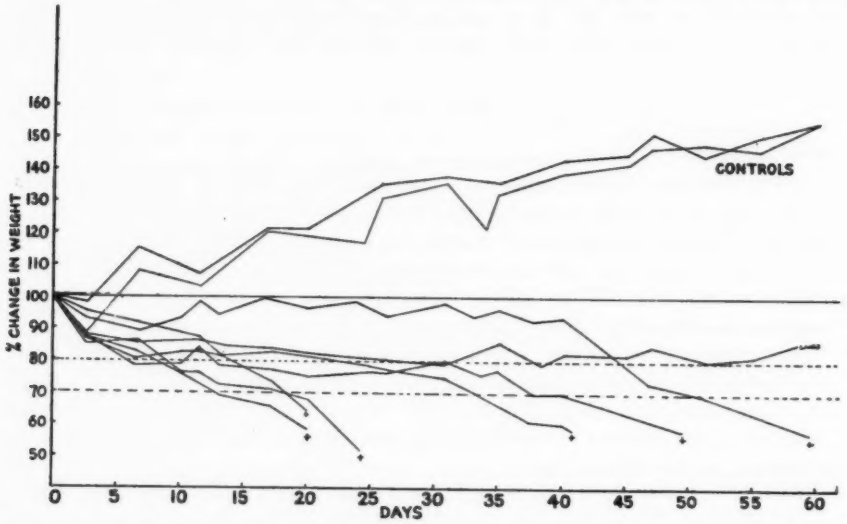

Fig. 1. Effect of 30 mEq of LiCl. per litre of drinking water on weight of young male rats (starting weight av. 120 grams). + = died.

p.c. during the first few days, an apparently stable condition, in which they ate and drank enough to maintain their reduced weight and were indistinguishable in their behaviour from normal rats. The condition was, however, unstable; sooner or later, usually within 2 to 4 weeks, they abruptly became ill, showed further weight loss and died within a few days with the same symptoms and about the same 40 p.c. weight loss as the rest (Fig. 1). The plasma lithium levels were during the pseudo-stable state about 3 mEq/1, and increased rapidly just before death. These

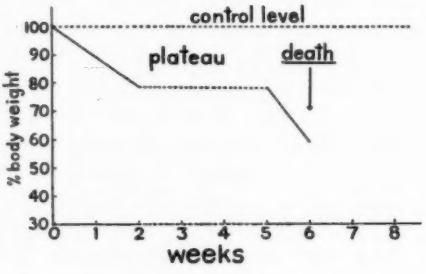

Fig. 2. Per cent. changes in body weight of adult rats on a toxic lithium concentration in the drinking water (30 mEq/l or more) showing the initial fall, the unstable "plateau" of varying duration and the abrupt final decline leading to death.

animals likewise recovered completely if they were put on pure drinking water. No persistent damage appeared to have been caused by the prolonged lithium ingestion.

With 20 mEq/1 of lithium chloride in the drinking water 95 p.c. of the animals survived for an indefinite time, up to over 2 years. Those who died did not show the symptoms of the animals dying on the higher lithium doses; most of them showed signs of intercurrent respiratory or abdominal illness. It could not be decided whether the incidence of death was significantly higher than in the lithium-free controls and possibly associated with a lithium-induced lowering of resistance to intercurrent disease or to stress. Adult animals put on this lithium dose did not show any pronounced changes in behaviour or sexual activity; they maintained their weight during months of lithium ingestion and were in all respects indistinguishable from the controls. It appeared that in some females the oestrous cycle as well as the period of gestation were slightly prolonged, but not sufficiently to be considered significant (oestrous cycle 4·18 to 4·24 days, gestation period 22 to 25 days). No transient or persistent anoestrous was observed. The plasma lithium levels were between 1·5 and 2 mEq/1.

With a content of 10 mEq of lithium chloride in the drinking water no difference between test and control animals could be observed for a period of over 2 years. The plasma lithium contents at this dose were below 1 mEq/1.

On these observations a content of 20 mEq of lithium chloride per litre of drinking water was selected as presenting a safe level of prolonged lithium medication, not likely to cause any somatic effects, and therefore suitable for the study of the effects of the ion on pregnancy.

Somatic disturbances accompanying lithium introduction.

The effects of changing the animals from lithium-free drinking water to water containing 20 mEq of lithium chloride per litre were not easily observable. Superficially there was no change in behaviour or appetite. Closer examination revealed transient changes, beginning almost immediately, but lasting not longer than 2 or 3 days. Table 1 gives the water and food intake on the first day of an experiment in which three sets of six animals were given pure water, water containing 20 mEq/1 of lithium chloride, and water containing 20 mEq/1 of sodium chloride respectively:

TABLE 1.

Composition of water	Mean fluid intake ml/100 gm.	Range of fluid intake ml/100 gm.	Mean food intake gm./100 gm. weight
Pure	12·81	11·0—17·0	7·5
20 mEq/LiCl	8·01	7·6— 9·0	6·9
20 mEq/NaCl	14·1	12·5—18·0	7·5

It appeared that, while the food intake was not appreciably changed, sodium chloride caused "thirst" while lithium chloride led to a reduced fluid intake. This may be due to its unpleasant taste, since the fluid intake returned to normal after a few days on the lithium solution. However, tabulation of the actual lithium intake gave an interesting picture. The above animals, on 20 mEq/1 of lithium chloride in their drinking water ingested, on an intake of 7·8 ml/100 gm., 156 micro Eq of lithium per 100 gm. of weight per day. Animals on 50 mEq/1 of lithium chloride in their drinking water and consuming 3·7 ml/100 gm. ingested 185 micro Eq of lithium chloride per 100 gm. per day, and those on 30 mEq/1 and fluid intakes of 5 to 6 ml/100 gm. ingested between 150 and 180 micro Eq per 100 gm. per day; that is, the amount of fluid taken was not so much controlled by thirst or taste as by the absolute amount of lithium ingested. The animals stopped drinking when they had ingested between 150 and 185 micro Eq of lithium. Furthermore, once the animals on 20 mEq/1 of lithium chloride in their water were adjusted to the diet, and their fluid intake rose again to 10 to 12 ml/100 gm. per day, they ingested significantly more lithium per day than those who died on contents of 30 to 50 mEq/1 of lithium chloride. Such a relation of lithium toxicity to the "hydration level" of the organism is possibly confirmed by the clinical observation that some patients who are well established on a prolonged high lithium dose show signs of toxicity during hot weather. The mechanism of lithium toxicity appears thus to be complex and not dependent on the magnitude of the dose alone. The question was not further investigated in the present work; the observations were merely taken as strongly suggesting that the initial, if transient, reduction in fluid intake cannot be accepted as being solely due to unpleasant taste, but must be suspected as demonstrating a more serious disturbance—even if the animals get adapted after a few days.

No observations were made indicating that, on a lithium chloride content of 20 mEq/1 in the drinking water, the initial disturbance extended for more than a few days. To be on the safe side, it was decided to allow for a period of 10 to 14 days of lithium ingestion before mating.

Normal pregnancies of lithium-treated females and controls (*Table* 2).

These series were conducted mainly to observe the incidence and progress of pregnancy, birth and lactation, and the health and progress of the young. The prospective mothers were selected and separated, the lithium-animals were put on drinking water containing 20 mEq of lithium chloride per litre for 3 to 7 weeks before mating was attempted, while the controls remained on pure water under the same conditions of environment and treatment.

None of the lithium-animals responded with any toxic signs or with noticeable behaviour changes.

Mating was carried out under the procedure described. The mean time for the animals to be served was 6 days varying equally in tests and controls. Pregnancy failed to occur after ascertained service in 10 out of 100 controls and in

6 out of 52 lithium-treated females. Some pseudo-pregnancies of lithium-treated females were observed, particularly in smaller, much handled series. To clarify the possible effect of lithium on the incidence of pregnancy, a slightly higher dose (25 mEq/1) was used in one or both sexes in smaller, separate series. The animals were put on this lithium dose 17 days before mating, and lithium-females were kept on lithium during pregnancy. The results were:

Males	Females	Pregnancies	Viable	Dead Young
water	water	5/6	39	1
lithium	water	4/6	32	none found
water	lithium	2/6	13	2
lithium	lithium	2/6	11	none found

TABLE 2.

Undisturbed pregnancies (findings at birth).

	Control (44)		Short-term Li (16)		Control (22)		Short-term Li (13)	
nr.	litter	viable	litter	viable	litter	viable	litter	viable
11	— —	— —	— —	— —	2	1	— —	— —
10	3	3	— —	— —	5	5	— —	— —
9	4	4	— —	— —	5	5	— —	— —
8	3	3	4	4	6	5	1	1
7	11	10	5	5	3	4	5	5
6	8	8	3	3	— —	— —	2	2
5	2	2	— —	— —	1	1	2	2
4	7	7	2	2	— —	1	1	1
3	3	2	2	1	— —	— —	1	— —
2	3	3	— —	— —	— —	— —	1	— —
1	— —	— —	— —	— —	— —	— —	— —	1
0	— —	2	— —	1	— —	— —	— —	1
sum	268	258	99	96	191	183	74	70
mean	6·09	5·86	6·19	6·00	8·68	8·52	5·69	5·38
S.D.	2·195	2·501	1·703	2·150	1·426	1·662	1·734	2·349

Legend to Tables 2 and 3.

All animals were healthy adult females; those of the Control Series were mated and kept under the same conditions as those of the Test Series (with exception of control series 1, 44 animals, which were much handled). The animals of the Short-term lithium series were on drinking water containing 20 mEq of LiCl per litre for 3-7 weeks before mating and during the whole time of pregnancy. The lithium-reared animals, 14, were fully grown offspring of the short term lithium series presented in Table 2, who had been kept on the same lithium content in their drinking water since birth. The figures listed under litter, corpora lutea, implants and viable give the number of mothers in whom the corresponding number of litter, corpora lutea, etc. was counted at birth in Table 2, and on biopsy in Table 3.

(In the series short-term Li, biopsy, nine animals were opened before the 16th day, when only corpora lutea and total implants could be reliably ascertained.)

TABLE 3.

Pregnancies inspected by biopsy on the 16th to 18th day.

	Controls (31)			Short-term Li			Lithium-reared (14)		
nr.	Corp.l.	Implants	viable	C.L.(31)	Imp.(31)	viable (22)	C.L.	Imp.	viable
13	2	— —	— —	— —	— —	— —	— —	— —	— —
12	2	2	— —	1	— —	— —	— —	— —	— —
11	9	7	6	2	— —	— —	— —	— —	— —
10	9	6	4	9	8	2	4	3	1
9	6	9	9	9	6	4	6	3	1
8	3	4	5	5	5	3	3	6	5
7	— —	1	2	5	6	6	1	1	3
6	— —	1	3	— —	2	4	— —	1	3
5	— —	— —	1	— —	2	2	— —	— —	— —
4	— —	1	1	— —	1	— —	— —	— —	1
3	— —	— —	— —	— —	1	1	— —	— —	— —
2	— —	— —	— —	— —	— —	— —	— —	— —	— —
1	— —	— —	— —	— —	— —	— —	— —	— —	— —
0	— —	— —	— —	— —	— —	— —	— —	— —	— —
sum	317	291	268	280	245	159	125	118	112
mean	10·22	9·39	8·65	9·02	7·90	7·23	8·93	8·42	7·29
S.D.	1·316	1·726	1·872	1·305	1·922	1·745	0·923	1·154	1·488

The lithium mothers in this experiment remained healthy during the entire pregnancy; they and the young were put on pure water after birth. No damage to mothers or litters was noted. Other series gave similar results.

In another series on a lithium content of 20 mEq/1, of normal females mated with lithium males 1 out of 15, of lithium females mated with normal males 1 out of 17, and of lithium females mated with lithium males 2 out of 7 failed to develop pregnancy although service had been ascertained.

The pregnancies and the act of birth itself appeared to be undisturbed. In some batches there seemed to be an increased tendency to eat the still-born or part of the litter. Occasionally the period of gestation was prolonged by one or two days, but all these phenomena were irregular and appeared to be more frequent in the more handled series. It was therefore decided to conduct the actual test series with as little handling and disturbance as possible. Table 2 gives the results of two series. No cause could be found to explain the low litters of control series I, or the fact that about one-fourth of these mothers had less than five young. In all other control series the mean of the litter was between 8 and 9, with very few litters numbering less than six.

The period of lactation was normal. The mothers showed no ill effects after the pregnancy. The effects of repeated pregnancies were not investigated.

All young, still-born or viable were carefully examined for malformations. None was found in this or any other series. The viable young, that is those who took to the mother and were alive on the day after birth, showed no significant difference in size or weight in the two series. However, the incidence of early death seemed to be slightly higher in the lithium series, possibly due to lack of maternal care. The lithium-young whose mothers were maintained on the same lithium content of the drinking water showed a slower gain in weight and growth. It appeared that some litters failed to put on weight during the first three days after birth, possibly suggesting reduced lactation, but this was seen in only 4 out of 19 lithium-females. The later mortality of the lithium-reared young was not different from that of the control young. After two or three months there was no longer any noticeable difference in size and weight between the young of the two series. The lithium-reared young were maintained on the same lithium content in the drinking water. They grew up to normal and healthy rats. The females were used for mating experiments reported below.

Opened lithium-treated pregnancies and controls (*Table* 3).

These series were conducted with the purpose of ascertaining by biopsy the number of corpora lutea, implantations and foetuses. To minimise the effect of handling and other environmental disturbances, both test and control animals were selected at the same time and left undisturbed for several weeks before mating was attempted. Biopsy was performed on the procedure described.

In preliminary experiments small groups of animals were opened on the 7th, 11th and 17th day of pregnancy. On the 7th day of pregnancy the implants were readily identifiable as swellings, a few millimeters across. It was thought that in the event of an early damage to the ovum implantation might fail to occur and manifest itself in the difference between the number of implants and corpora lutea counted. However, on a lithium chloride content of 20 mEq/1 no useful observation was gained. On the 11th day the swellings were larger, but it was difficult to distinguish early resorptions from viable foetuses. On the 16th to 18th day the viable foetuses were clearly visible through the uterine wall, while resorption sites were identifiable as small dark red, opaque, clot-like masses or as white placental scars. On these findings the 16th to 18th day of the pregnancy was chosen for subsequent laparotomies. Like the unopened animals the rats littered on the 22nd to 24th day of pregnancy. The lithium animals did not show lower resistance to the operation. Occasionally a foetus found viable at biopsy was subsequently still-born; the incidence was equal in test and control animals. In two instances, one test and one control, the whole litter was still-born, although these foetuses were alive on the day of biopsy. The normal litter numbered nine, the lithium litter seven young. (These animals are included in the records presented.) No malformation or other cause of death

could be noted. In two instances there was one implant more than corresponded to the number of corpora lutea counted. This may have been due to twinning.

Opened short-term (3-7 weeks) lithium pregnancies. The reduction in corpora lutea was highly significant (about 15 p.c.). No differences in the degree of reduction of the mean-values of corpora lutea, implantations or viable foetuses were observed whether the mothers had been on lithium for 2 to 3 or for over 7 weeks. No return to normal values was ever noted. In several smaller batches (not recorded) it seemed that the number of implants as compared with that of the corpora lutea, was decreased and that the number of resorptions was increased. In the bigger, possibly less disturbed series the differences were still suggestive, but not significant. There was no difference between tests and controls with regard to the aspect and size of the corpora lutea, resorption sites or developing foetuses. No observations were made which would indicate that there was, at least during the second half of the pregnancy, any difference in the fate of the normal and of lithium-bred young. Neiher was there, despite the operation, any difference in the survival rate of those young who were viable on the day after birth. No correlation between the lack of implantation or the tendency to resorption and the number of corpora lutea present could be detected either in the normal or in the test series. The later development of the litter was identical to that of the unopened pregnancies.

Opened lithium-reared pregnancies. The reduction of corpora lutea was the same as in the short-term lithium series and equally significant. For this small series, female rats were used who during their whole life had been on drinking water containing 20 mEq of lithium chloride per litre. The animals were born from mothers who before and during their pregnancy were on lithium. The age of these animals at the time of the beginning of the experiment was 6 to 7 months. At this time their weight was not significantly different from that of normal females of the same age and the animals were in all aspects indistinguishable from normal rats.

The animals were carefully observed during pregnancy and lactation, but no significant differences from the previous series or the controls could be noted. There were no ill effects on the mother during or after birth, and no malformation of the foetuses could be found. Neither was there any undue effect of the operation.

Evaluation of results.

Many of the observations recorded were irregular, though marked enough not to be ignored; some were observed in some series, but were absent in others. The counts of still-born and viable in normal pregnancies were unreliable, since the counting was done several hours after birth and did not necessarily correspond to the actual numbers. Statistical evaluation must, therefore, be confined to the counts of corpora lutea, implants and fully developed, viable foetuses as recorded in biopsies on the 16th to 18th day of pregnancy. These observations were:

TABLE 4.

	Controls	Short-term Li	Lithium-reared	Pooled lithium
Number of mothers	31	31	14	45
Corpora lutea: range of counts	8—13	7—12	7—10	7—12
Mean	10·22	9·03	8·93	8·95
Standard Dev.	1·316	1.303	0·923	1·16
Implants: range	4—12	3—10	6—10	3—10
Mean	9·39	7·90	8·43	8·1
S.D.	1·726	1·922	1·16	1·73
Viable 16/18th day: Range	4—11	1—10	4—10	1—10
Mean	8·65	7·23	7·29	7·25
S.D.	1·872	1·745	1·49	1·65

These values do not significantly differ from those in other series not recorded, such as the counts in corpora lutea or implants on mothers opened on the 7th or 11th day of pregnancy.

It will be noted that the individual numbers of corpora lutea, implants or viable 17th day foetuses are not always symmetrically distributed around the mean. The degree of skewness (g_1) was therefore calculated to establish whether the deviations from the symmetrical Gaussian distribution were significant enough to interfere with the comparison of the results. The point may be of importance in view of the evaluation of the results for the clinical use of lithium. The values for g_1 were:

	Corp. lut.	Implants	Viable 17th day.
Control g_1	+0·0074	−1·0698	−0·6916
(t)	(+0·0175)	(−2·58)	(−1·645)
Short-term Li g_1	+0·3881	−0·0813	−0·3884
(t)	(+0·923)	(−1·934)	(−0·798)
Li reared g_1	−0·5472	−0·3229	−0·4188
(t)	(−0·906)	(−0·5347)	(−0·6934)

It is seen that only the control implants are significantly skewed ($p < 0·01$).

When the g_1 values of the individual series were compared with each other it was found that the differences between their skewness was not significant, except for: control implants vs short-term corpora lutea ($t = 2·451$, $p = 0·02$) which is distinctly significant, and control corpora lutea vs control implants ($t = 1·811$, $p = 0·07$) which is barely significant. That is, the negative skewness of the control implants was significantly different from the skewness of the two positive values, but there was no significant difference between the individual negative skewness of the other series.

If both short-term lithium and lithium-reared results were pooled, the corpora lutea distribution was found to be symmetrical. The pooled implants showed significant negative skewness ($p = 0{\cdot}01$), but the difference between their skewness and that of the viable 16th day foetuses was not significant. Furthermore, the variance ratio test showed the variances of these two series to be not significantly different.

On these findings it appeared to be justified to calculate Student's t and probability values on the assumption of symmetrical or near-symmetrical distribution. The results are presented with the reservation that, *whenever implants are considered caution must be observed since some disturbance in their distribution as compared with that of the other groups appears to be present.*

Student's t and probability values of corpora lutea, implantations and viable 17th day foetuses calculated on the assumption of symmetrical distribution of the data are presented in Table 5.

TABLE 5.

Short-term lithium/Controls.

	t	P
Corpora lutea	3·527	0·001<P<0·01
Implantations	3·145	0·001<P<0·01
Viable 17th day	2·740	0·001<P<0·01

Lithium-reared/Controls

	t	P
Corpora lutea	3·267	0·001<P<0·01
Implantations	1·851	0·1
Viable 17th day	2·341	0·02

Short-term lithium/Lithium reared

	t	P
Corpora lutea	0·263	0·8
Implantations	1·015	0·3
Viable 17th day	0·102	0·9

The formulae used for calculating the degrees of skewness, g_1 and its significance t were:

$$g_1 = \left(\frac{K_3}{\sqrt{K_2^3}}\right) \qquad \text{where} \qquad K_3 = \frac{N.\,\Sigma x^3}{(N-1)(N-2)}$$

$$K_2 = \frac{\Sigma x^2}{N-1}$$

$$t = \frac{g_1}{Sg_1} \qquad \text{where} \qquad Sg_1^2 = \frac{N(N-1)}{(N-2)(N+1)(N+3)}$$

The figures show that the reduction of the number of corpora lutea in both lithium series, as compared with that of the controls, was highly significant, but that there was no significant difference between the number of corpora lutea in the short-term and the lithium-reared animals. There was, however, some though not significant difference in the chance of the ova (counted as corpora lutea on biopsy) becoming fertilized, implanted and developed. The per cent. probabilities were:

	Controls	Short-term Li	Li-reared	Total Li
Implantations	92 p.c.	87 p.c.	95 p.c.	91 p.c.
Viable 17th day	84 p.c.	76·5 p.c.	81·4 p.c.	81·5 p.c.

The percentage of implantations that lead to viable foetuses was equal in both lithium series, 87 p.c. compared with 92·5 p.c. in the controls.

The findings suggest that the main effect of lithium ingestion under the conditions of the experiment was exercised on ovulation, that is before mating; and that there was little if any effect of lithium during the period from implantation to viable foetus. The percentage of implantations was reduced in the short-term lithium series, but increased in the lithium-reared animals, with reduced SD, but the latter series was possibly too small to allow definite conclusions. In both lithium series a slightly higher percentage of the implants were resorbed.

If the findings on the short-term lithium and the lithium-reared animals were pooled, only the 15 p.c. reduction in the number of corpora lutea remained significant.

DISCUSSION.

The present observations were made on female rats, maintained on a constant concentration of lithium chloride in their drinking water, and mated with healthy males. The lithium concentration, 20 mEq/1, was near the upper limit which was indefinitely compatible with the life and health of the animals. It is comparable to the highest doses given temporarily to hospitalised patients (plasma lithium level about 2 mEq/1). Two highly significant observations were made: (1) a constant (approximately 15 p.c.) reduction in the number of corpora lutea found on biopsy, and (2) the absence of any malformations in the dead or live young, or of any damage to the mothers.

Since the reduction in the number of corpora lutea was observed after only 2 to 3 weeks of lithium ingestion, the drug must be assumed to act quickly, probably on the developing follicle. No indications were found to explain the mode of its action. Lithium is used in the treatment of conditions of psychomotor excitement; in many of these conditions its use is comparable with that of salt-depleting agents, diuretics, Diamox, ammonium chloride, etc., and may be explained by an effect of the ionic content and the hydration of the cell. Following McQuarrie's (1929) work on epileptic children such a disorder has been

established in a number of pathological conditions. In some of these conditions, like post-puerperal psychosis and premenstrual tension, the primary disturbance appears to be hormonal. If lithium affects hormonal mechanisms, the observed reduction in folliculation may be understandable.

Selye (1950) states: "Experiments in rats suggest that the characteristic response of the female sex organs to systemic stress manifests itself mainly by ovarian atrophy and more or less permanent anoestrus . . . upon continuous exposure to stressors the ovary returns towards normal and vaginal cycles reappear as soon as adaptation becomes adequate . . . Follicle maturation and corpora lutea formation may continue in animals which are not especially severely damaged. Under these conditions usually only one or two (instead of the normal 10-12) follicles are transformed into corpora lutea at each ovulation." Higher lithium doses showed clearly the acute effects of an initial high lithium impact and the subsequent formation of a temporary, unstable "plateau" of adaptation (although no early irreversible damage appeared to have been caused), but the animals on the lower dosage of 20 mEq/l showed neither this "plateau" effect nor an initial transient anoestrus nor a later return from the reduced level of corpora lutea to normality. The corpora lutea formation of the lithium-reared females was almost the same as that of animals having been on lithium for only 3 to 4 weeks and remained of the same magnitude for the period of observation. If there was a systemic lithium damage, it did not seem to be liable to adaptation or compensation.

Malformations may be caused by a series of compounds if they are administered either throughout pregnancy, or given in higher concentrations at critical stages of pregnancy. Lithium chloride (2 mg. twice daily i.p. for 2-3 days) increased death rate of the young if injected between the 2nd and 7th day of pregnancy in rats or mice, but not on later injection (Bass, 1951). The author does not record the occurrence of malformations or increased death rate. The present observations suggest that there was no or only very little lithium damage after established implantation.

Some observations were not statistically significant as to their magnitude, occurrence or distribution, but frequent enough not to be ignored. They were:

(1) Failure of pregnancy to occur after ascertained service, or pseudo-pregnancies with corpora lutea persisting up to the 11th day; (2) increased disturbance of implantation or early resorptions; (3) increased number of still-born and increased early mortality; (4) increased tendency of the mother to eat the young; (5) reduced lactation, with initial reduction of the rate of growth.

Points (3) and (5) may be understood as being due to a general somatic effect of the lithium ion on the mother, possibly not connected with the highly significant reduction of corpora lutea.

Points (1) and (2) may be explained by a direct lithium effect on the ovum. In the control series about 9 p.c. of the ova are "doomed", that is, they do not

lead to implantations; another 9 p.c. perish and get re-absorbed before birth. If these lost ova are not doomed by hazard, but through a reduced viability, manifesting itself in a reduced facility to be fertilized and to cause implantation, then the additional effect of lithium may increase the number of "doomed" ova, and further increase the difficulties they meet in their biological path. This may explain the (tentative) observation of a higher implant rate in the lithium-reared mothers, if one assumes that the damage to the ovum is done during the follicular development and that the less viable do not result in ripe follicles. Those which survive folliculation are strong enough to be implanted.

However, the striking effect on incidence of pregnancy and number of the litter observed on only slightly higher lithium doses, particularly if both parents were on lithium-containing drinking water, may mean that some of the mothers, although apparently in good health, were actually just at the margin of stable adaptation. The observations (1) and (2) would then be due not only to an impairment of the development of the follicle, but also of the uterine response to the fertilized egg, or to the effect of a lithium-containing medium on the spermatozoa, or to reduction of service.

A different explanation may apply to points (1), (3) and (4): lithium may have a possibly non-specific and indirect effect on the factors guiding behaviour patterns of the mother, resulting in a greater dependency on environment. If it impairs the mating urge, inadequate service may result in either complete failure of pregnancy or in pseudo-pregnancy, despite the fact that some spermatozoa are found in the vaginal smear. If the nursing urge is impaired, the young may not receive adequate early attention, resulting in an increase of "still-born" and early deaths. Both these effects may be potentiated by slight and untraceable disturbances in the environment, explaining their irregular occurrence.

The main effect of lithium, however, manifested itself in a significant reduction of the number of the corpora lutea in the lithium-treated females. It appears justified to consider that this reduction was caused by an inhibitory effect of the ion on the development of the follicule and the ovum. The investigation has therefore been extended to a study of the effects of lithium on other rapidly growing cells, namely, carcinomatous tissue.

Obviously, the question arises to what extent the observations on rats are relevant to the management of human pregnancies of patients under prolonged lithium medication. The total absence of malformations seems strongly to suggest that no lithium damage to the foetus need be feared. Supplemented food and early weaning may have to be considered to avoid unnecessary lithium ingestion. The possible effects or dangers of high lithium ingestion by the mother directly before and during the first months of pregnancy are more difficult to assess. Grave disturbances of the menstrual cycle and of menstruation have so far not been observed in human patients and, whether there is increased

incidence of non-ovulatory menstruation is not known. Neither is there any information whether the reduced rate of implantation and the increased number of early resorptions in the rat are paralleled in the human by early abortions. Until such information is available it may be safest to interrupt lithium ingestion once a pregnancy is established, and, if necessary, to re-introduce it cautiously during the second half of the pregnancy, if symptoms of psychomotor instability appear.

SUMMARY.

The effects of prolonged lithium ingestion on pregnancy were investigated in the rat. Lithium chloride was administered to adult female rats in a concentration of 20 mEq per litre of drinking water. This dose was just subtoxic; apart from slight initial disturbances, it caused no effects on health or behaviour in the adult animals over a period of up to two years. The mating experiments were conducted in series under the same conditions as control series.

In most series the number of corpora lutea, total implants and viable foetuses was ascertained by biopsy on the 16th to 18th day of pregnancy.

The main effect of prolonged lithium ingestion was a drop in the number of corpora lutea by about 15 p.c. This effect was present after 3 to 4 weeks of lithium administration; it was of about the same order in lithium-reared animals and remained constant during the time of lithium ingestion.

No malformations were found in any of the live or still-born young. No damage to the mothers was observed.

The young, if maintained on the same lithium concentration in the drinking water, showed slightly slower growth, but developed finally into adult rats indistinguishable from normal rats.

A number of inconstant and statistically non-significant disturbances was observed. They suggest a further, if slight, lithium damage occurring between folliculation and safe implantation, and independent of that causing the reduction in corpora lutea.

The possible significance of the results with regard to the pregnancy of lithium-treated human females is discussed. It is suggested temporarily to interrupt lithium medication during the first third of pregnancy and cautiously to restart it later on, if required.

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THE EFFECT OF POTASSIUM ON THE RESPONSE OF THE ISOLATED HEART TO STROPHANTHIN-G

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Although the mechanism underlying the action of cardiac glycosides on electrolyte transport across cell membranes has not been clearly established, many investigators have recorded a direct effect of glycosides upon potassium exchange rates.

Potassium loss from isolated myocardium following digitalis therapy was recorded by Cattell and Goodell (1937), Wood and Moe (1938), Calhoun and Harrison (1931). More recently Rayner and Weatherall (1957) using radioactive tracers observed reduced rates of influx of potassium in right and left rabbit auricles following treatment with digoxin and ouabain; they noted a concomitant gain in sodium. Regan, Talmers and Hellems (1956) observed an abrupt rise in arterial potassium in anaesthetized dogs following treatment with acetyl strophanthin at therapeutic levels. Joyce and Weatherall (1955) found potassium influx in human erythrocytes greatly reduced after treatment with digoxin and k-strophanthoside, thus confirming the results of Schatzmann (1953) and Glynn (1955).

In contrast, Gonlubol, Siegal and Bing (1956) using catheterization of the coronary sinus in human hearts failed to find any significant change in the sodium-potassium balance of the heart following infusion with cedilanide and suggested that the rise in plasma or perfusate potassium concentrations reported by other workers was due to a peripheral action of the drug. In isolated rabbit heart preparations Hagen (1939) recorded a slight increase in myocardial potassium concentration at therapeutic levels of digilamide and a marked decrease in potassium only at toxic levels.

This present investigation was carried out to determine whether changes in potassium exchange rates produced any significant change in the magnitude and duration of the positive inotropic effect of strophanthin-G on the isolated toad heart. In some experiments the rate of K^+ transport was limited by varying the potassium concentration of the perfusate, in others by the addition of quinidine sulphate to the preparation (Armitage, 1957) so that observations on the

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mode of action of quinidine sulphate on the isolated heart together with its effect upon the positive inotropic response were made.

By these means it was established that the action of the cardiac glycosides on potassium transfer was linked in some way with the metabolic and positive inotropic effects produced by these drugs.

Materials and Methods.

In this series all experiments were made at 25·0 ± 0·05° C. using the isolated spontaneously beating heart of unselected summer toads (*Bufo marinus*) as previously described (Nayler and McKelvie, 1956). The perfusion apparatus was oxygenated for ten minutes and a further thirty minutes allowed for temperature equilibration at 25·0° C. before observations were commenced.

Unless otherwise stated, standard perfusion fluid of the following composition was used throughout the four hours' perfusion:

NaCl	115 mM/litre
$NaHCO_3$	20·6 mM/litre
NaH_2PO_4-$2H_2O$ ...	3·0 mM/litre
$MgSO_4$ $7H_2O$ ...	1·2 mM/litre
Glucose	16·5 mM/litre
KCl	3·2 mM/litre
$CaCl_2$	1·3 mM/litre

In those preparations in which the potassium concentration of the perfusate was varied correct tonicity was maintained by the addition or deletion of glucose.

Potassium concentration.

The action of strophanthin-G[2] (1×10^{-5} final conc.) when added to hearts perfused with Ringer solution containing potassium at 1·6, 2·0, 2·4, 3·2 and 4·8 mM KCl/litre was recorded, six experiments being performed at each potassium concentration. Hearts were perfused for one hour prior to and three hours following the addition of the glycoside during which time observations on oxygen uptake, carbon dioxide output, drops per beat, beats per minute and maximum aortic pressure were recorded at fifteen-minute intervals.

Control experiments were made at each potassium concentration used above.

Quinidine sulphate.

The action of quinidine sulphate[3] (1×10^{-5} and 1×10^{-6} final conc.) on the isolated heart perfused with standard perfusion fluid was recorded as above, six experiments being performed at both concentrations and observations recorded during the initial hour and three hours following the administration of the drug.

In a further series of experiments the action of strophanthin-G (1×10^{-5} final concentration) on hearts initially perfused with Ringer solution containing quinidine sulphate (1×10^{-5} and 1×10^{-6} final conc.) was noted. Six experiments were performed at both quinidine concentrations and observations made regularly throughout the four hours' perfusion as usual.

RESULTS.

Potassium series.

The magnitude and duration of the positive inotropic response produced by strophanthin-G at the concentration used in these experiments varied according

[2] Strophanthin-G as Ouabaine Arnaud, Laboratoire Nativelle Product.
[3] Quinidine sulphate as Quinicardine, Laboratoire Nativelle Product.

to the concentration of potassium in the perfusate $[K_o]$[4], the maximum response being recorded when $[K_o]$ was 3·2 mM/litre. The mean maximum aortic pressures recorded in the series are shown in Fig. 1, where it is apparent that

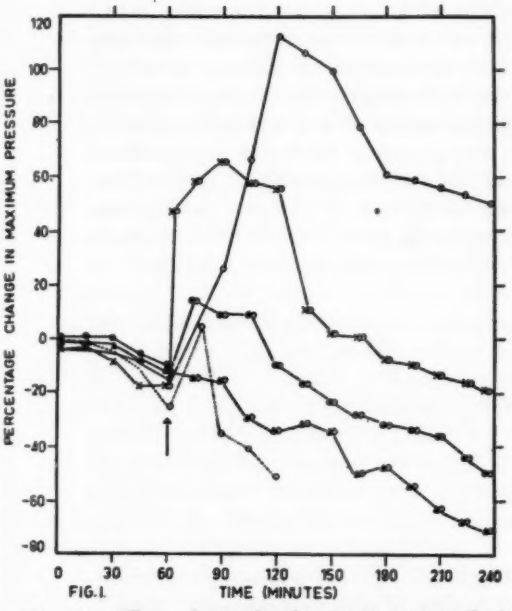


Fig. 1. Effect of strophanthin-G (1×10^{-5} final conc.) on maximum aortic pressures of hearts perfused with Ringer containing varied potassium concentrations ($[K_o]$)—see key Figs 1 and 2. Mean results of six experiments are shown at each $[K_o]$ value.

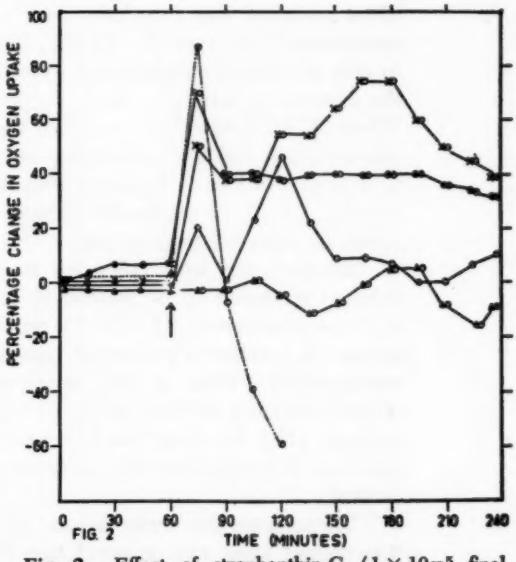


Fig. 2. Effect of strophanthin-G (1×10^{-5} final conc.) on oxygen utilization of hearts perfused with Ringer containing varied potassium concentrations ($[K_o]$)—see key Figs. 1 and 2. Mean results of six experiments are displayed at each $[K_o]$ value.

above or below the critical $[K_o]$ of 3·2 mM/litre the increased aortic pressures recorded following the addition of the glycoside were sub-maximal. At $[K_o]$ 4·8 mM/litre the positive inotropic effect as indicated by augmented aortic pressure was completely suppressed. Changes in beat rate and stroke volume (drops per beat) followed a pattern similar to that displayed by the pressure records in that the maximum effect of the glycoside was recorded when $[K_o]$ was 3·2 mM/litre.

KEY—FIGS. 1 & 2

K_o (mM/LITRE)	
1·6	
2·0	
2·4	
3·2	
4·8	
STROPHANTHIN-G (1×10^{-5})	

[4] $[K_o]$ represents the concentration of potassium in the perfusate.

Increased oxidative metabolism following the addition of strophanthin-G to the perfusate (indicated by an altered rate of oxygen uptake) displayed a dependence upon [K_o] similar to that observed in the aortic pressure records—see Fig. 2, where the mean rates of oxygen utilization have been expressed as the percentage change relative to the levels recorded throughout the initial hour's control perfusions. At [K_o] 1·6 mM/litre the small transient positive inotropic effect recorded was associated with a sharp but temporary increase in oxygen uptake—see Figs. 1 and 2. At [K_o] 2·0 and 2·4 mM/litre the maximum increase in rate of oxidative metabolism exceeded that noted at 3·2 mM/litre, although the increase in maximum aortic pressure was greater at the latter concentration. When [K_o] 4·8 mM/litre was used the addition of strophanthin-G at the above concentration failed to effect any increase in the rate of oxidative metabolism. Comparison of Figs. 1 and 2 suggests that when [K_o] was 3·2 mM/litre strophanthin-G (1×10^{-5}) maximally increased the aortic pressure with a minimal increase in oxidative metabolism.

Similarly the time taken for the inotropic response to be maximally developed appeared to be related to [K_o] levels. When low [K_o] values were used strophanthin-G (1×10^{-5}) rapidly produced its maximum effect, but at higher [K_o] values a prolonged time interval elapsed before the peak response was reached. Thus, at [K_o] levels of 1·6 and 2·0 mM/litre fifteen minutes elapsed after the addition of the glycoside before the maximum change in aortic pressure (Fig. 1), drops per beat and beat rate were recorded, whereas at [K_o] values of 3·2 mM/litre sixty minutes elapsed before the maximum effects were evident.

Throughout this series it was noted that the external potassium concentration played some rôle in regulating the toxicity of the glycoside—see Table 1. At low [K_o] values strophanthin-G (1×10^{-5}) showed marked toxic effects—toxicity being indicated by persistent arrhythmias often extending into complete cardiac arrest.

TABLE 1.

mM KCl/litre	1·6	2·0	2·4	3·2	3·6	4·8
No. of exp.	6	6	6	6	6	6
No. of toxic responses	6	3	2	0	0	0

Quinidine sulphate.

In Fig. 3, A-F, the results obtained following the addition of quinidine sulphate (1×10^{-5} final conc.) to a series of six hearts initially perfused in standard Ringer solution are compared with those obtained from a similar series of control experiments using normal Ringer throughout. At this concentration quinidine caused a decrease in the beat rate (Fig. 3 C) and number of drops per beat

(Fig. 3 B) together with a steady fall in the maximum aortic pressure (Fig. 3 D). These changes resulted in a decreased work output of the heart (Fig. 3 E) which was associated with a depressed level of oxidative metabolism (Fig. 3 A), but no significant change in respiratory quotients. The overall result of these changes was a well-sustained fall in efficiency (Fig. 3 F).

Quinidine sulphate (1×10^{-6} final conc.) failed to produce any significant change in beat rate, drops per beat, maximum aortic pressure or rate of oxygen uptake of the isolated heart used in this preparation.

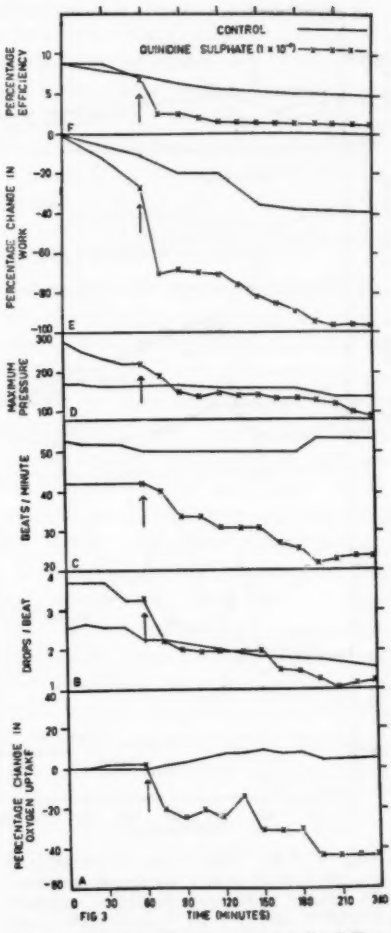


Fig. 3 A-F. Observations made following the addition of quinidine sulphate to hearts perfused with normal Ringer—mean results of six experiments.

Quinidine plus glycoside.

When strophanthin-G (1×10^{-5}) was added to hearts perfused throughout the initial control period with quinidine enriched Ringer solution (final conc. 1×10^{-5}) the positive inotropic response normally recorded following the addition of the glycoside at this concentration was completely suppressed. The results obtained from six such preparations are displayed in Figs. 4 (A-D) and 5, where they are compared with the positive inotropic effect elicited by strophanthin-G at the above concentration from a series of six preparations perfused with normal Ringer solution. Whereas strophanthin-G alone caused a marked and sustained increase in work output of the isolated heart, together with augmented oxidative metabolism, the presence of quinidine sulphate (1×10^{-5}) apparently completely blocked the inotropic response (Fig. 5) and increased oxygen uptake normally associated with it (Fig. 4 A).

When the dose level of quinidine was reduced to 1×10^{-6} final conc., strophanthin-G (1×10^{-5}) produced a positive inotropic effect and increased oxidative metabolism but of lesser magnitude than that due to strophanthin-G alone (Figs. 4 A-D, and 5).

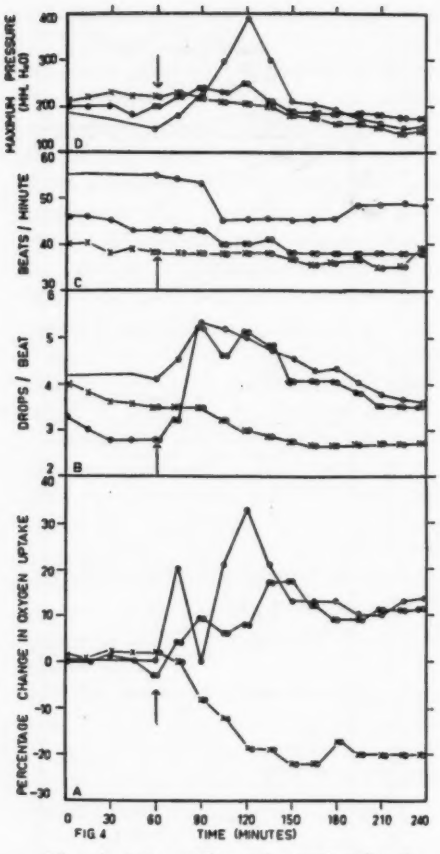
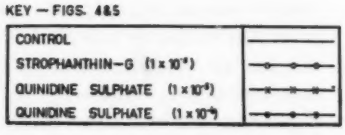


Fig. 4 A-D. Effect of strophanthin-G (1×10^{-5} final conc.) on hearts perfused with Ringer containing quinidine sulphate (1×10^{-5} and 1×10^{-6} final conc.)—see key Figs. 4 and 5. At both quinidine conc. the mean results of six experiments are shown.



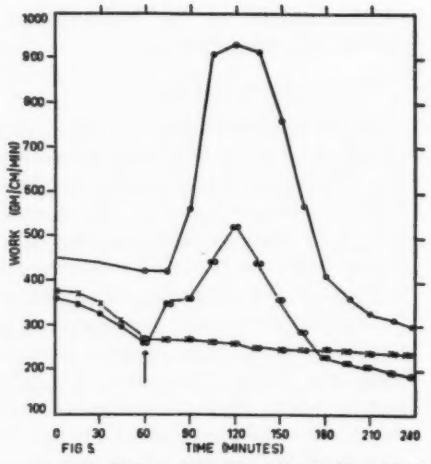


Fig. 5. Effect of strophanthin-G (1×10^{-5}) on work output of hearts perfused with Ringer solution enriched with quinidine sulphate (1×10^{-5} and 1×10^{-6} final conc.)—see key Figs. 4 and 5. Mean results of six experiments at both quinidine concentrations.

DISCUSSION.

Throughout these experiments the positive inotropic response produced by strophanthin-G was rapidly apparent in those hearts perfused with low potassium Ringer, where the loss of K^+ from the myocardium would be facilitated and re-entry of K^+ depressed. As well as the time of onset, the rate of loss of K^+ from the myocardium apparently governed, in part at least, the magnitude,

duration and toxicity of the response and the rate of increased oxidative metabolism normally associated with the positive inotropic effect (Nayler, 1957).

The positive inotropic effect was rapidly apparent in hearts perfused in low K^+ Ringer possibly because less energy was required to extrude a critical concentration of potassium from the myocardium. Since this response was greatly reduced in magnitude and duration when compared with that normally recorded at $[K_o]$ 3·2 mM/litre it is probable that a critical time interval must exist between the administration of the glycoside and the development of the maximum inotropic effect, this time interval being related to the rate of loss of K^+ from the myocardium.

At higher potassium levels efflux of K^+ would be more difficult and a greater energy supply needed to extrude the critical K^+ concentration. This condition was reflected in the delayed and reduced inotropic effect recorded at potassium concentrations exceeding 3·2 mM/litre.

These results of this series differ from those of Garb and Venturi (1954) who, using isolated cat papillary muscle preparations, failed to note any significant modification of the positive inotropic action when the potassium concentrations of their perfusion fluid was varied between 3·5 and 8·5 mM/litre. However, these workers did record that the onset of toxic arrhythmias produced by ouabain was delayed or prevented at the higher potassium concentrations. This conclusion has been reached by many investigators using a variety of test methods (Baker, 1947; Lown *et al.*, 1951; Friedman and Bine, 1947). The last-named workers found that orally administered potassium temporarily abolished the E.C.G. manifestations of digitalis intoxication. The results reported in this present study support these conclusions, since no evidence of toxicity was recorded at the higher potassium levels. The sharp rate of increase of oxidative metabolism recorded following the addition of the glycoside to hearts perfused with low potassium Ringer may be associated with its toxic effects in these preparations.

In general, the inhibition of the positive inotropic effect by pre-treatment of the isolated heart with suitable concentrations of quinidine confirms the conclusion reached from those experiments in which the potassium concentration was directly varied, i.e. the normal inotropic effect as well as the toxic effects of the glycoside are at least partially controlled by the rate of loss of potassium from the myocardium.

Whereas acetylcholine facilitates the passage of K^+ across cell membrane (Armitage *et al.*, 1957; Harris, 1956) antimalarial substances including quinidine have the reverse effect and lower the permeability of the myocardium to these ions. In this series pre-treatment of the isolated hearts with quinidine (1×10^{-5}) apparently reduced the permeability of the membrane to K^+ to such an extent that the glycoside was unable to affect the normal loss of K^+ from the myocardium. The inotropic response and associated augmented oxidative metabolism were then completely suppressed. At the lower dose level

(1×10^{-6}) the rate of potassium efflux was only partially restricted so that an inotropic response of lesser duration and magnitude, together with a small increase in oxygen uptake was recorded. Some workers (Chatterjee, 1954; Dunn, 1955) have suggested that acetylcholine may play some part in the digitalis response, so that a changed acetylcholine turnover rate results in a secondary change in permeability of cardiac cells to K^+. Holland *et al.* (1952) have shown that potassium loss from guinea pig auricles is related to cholinesterase activity. This may explain the effect of quinidine on the inotropic response in this series.

Although contradictory evidence does exist (Gonlubol *et al*, 1956) the bulk of the literature relating to the ionic exchanges associated with the inotropic effect of the glycosides indicates that these drugs limit the rate of K^+ influx (Joyce and Weatherall, 1955; Glynn, 1957). This inhibition of potassium transport results in a changed intracellular sodium as well as potassium pattern, since the rates of influx and efflux of these two ions appear to be fundamentally relater (Edwards and Harris, 1957).

It is well established that the contractile matter of the heart is very sensitive to the intracellular ionic composition (Hajdu, 1953). Stutz *et al.* (1954) studying the behaviour of extracted heart muscles stressed the importance of the ionic gradient between muscle cell and the surrounding medium, and suggested that the intracellular ionic atmosphere determined the behaviour of actin and myosin towards each other in any particular system. Since the membrane regulates the intracellular ionic concentrations any factors which influence the selective permeability of the cell membrane will influence the ionic gradient between muscle cell and the surrounding media and so the contractile force developed. A similar conclusion was reached by Szent-Gyorgyi (1952) and Newbold and Rose (1957). The experiments in the present study confirm these conclusions and emphasize the fact that physiologically significant data relating to factors controlling contractile systems and the forces developed in such systems can only be obtained from studies made on intact muscles placed in carefully controlled ionic media.

SUMMARY.

The positive inotropic effect of strophanthin-G was measured at external potassium concentrations [K_o] varying from 1·6 to 4·8 mM/litre.

At low [K_o] levels the inotropic response was rapidly apparent but of reduced magnitude and duration and accompanied by a marked increase in oxidative metabolism.

At higher [K_o] levels (3·2 mM/litre) the inotropic response was delayed but of greater strength and duration than that recorded at lower [K_o] values. The increased level of oxygen uptake following the addition of the glycoside at high [K_o] levels was less than that recorded at low [K_o] levels. When [K_o] of 4·8 mM/litre was used no inotropic response was recorded.

Quinidine sulphate (1×10^{-5}) depressed the work output, efficiency and oxidative metabolism of the heart without any change in respiratory quotient values.

The inotropic response was completely blocked by quinidine sulphate (1×10^{-5}) and partially when the dose was reduced to 1×10^{-6}.

The toxicity of strophanthin-G was greatest at low [K_o] concentrations.

Acknowledgment. I wish to thank Dr. T. E. Lowe for his interest in and encouragement with this work.

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ETHER SOLUBLE COMPOUNDS ASSOCIATED WITH THE PLUMAGE OF BIRDS: A STEROID CONSTITUENT

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In addition to water soluble compounds associated with the epidermis and the keratinous appendages of the integument, substances soluble in organic solvents are also present. Material for studying the latter substances was obtained by extracting shed skin, fur and particularly feathers (Bolliger and Gross, 1956, 1957). In the present investigation the plumage of fowl, pigeons, ducks, geese and seagulls was investigated.

MATERIALS AND METHODS.

Freshly plucked, clean feathers were immersed in ether and left for several days. This process was repeated three or four times and the combined, filtered ether extracts evaporated to dryness. The residue, the feather lipids (yields are summarised in Table 1) were analysed for acid number, iodine number, melting point and total nitrogen by standard procedures and the remainder taken up in hexane. A small insoluble portion was filtered off. This hexane solution, which was of a yellow colour, was passed through a column of aluminium oxide (B.D.H. for chromatographic analysis). The dimensions of the column were adjusted so that the effluent was colourless, i.e., all the yellow colour remained on the column after washing with hexane. With larger amounts of material it may be more convenient to use more than one column.

TABLE 1.

Constants and composition of feather lipids.

	Fowl	Pigeon	Duck
M.p°C.	57	55	27-72
Iodine No.	22	3	36
Acid No.	7	36	13
Total N (p.c.)	0·38	0·30	0·03
Feather lipids (p.c. dry feather wt.)	1·2 (0·8)	1·7	2·5
Fraction 1 (p.c. dry lipid wt.)	45 (54)*	33	54
Fraction 2 (p.c. dry lipid wt.)	8 (10)	6-10	10
Plumin (p.c. dry lipid wt.)	30 (45)	25	24

* Figures in brackets refer to feather lipids of castrated roosters.

[1] Working under a grant from the National Health and Medical Research Council.

Further elution with benzene gave a solid, recovered by evaporation and rechromatographed on aluminium oxide in hexane solution. Two subfractions were obtained by first washing the column with hexane and then with benzene. The small amount of solid residue recovered from the hexane solution was recrystallised from ethanol, and the benzene-soluble solid was further purified by recrystallisation from methanol.

A third fraction was obtained by elution with alcohol and a final fraction by elution with glacial acetic acid. The latter was then evaporated to dryness, the solid residue was extracted several times with boiling ethanol, and finally fractionally recrystallised from methanol.

Alternate method for separation. A 2 p.c. solution of feather lipids in ether was extracted three times with 1 p.c. aqueous potassium hydroxide, and finally with water. On acidification of the combined aqueous layers fatty acids separated out and could be filtered off. The substances in the ether layer were separated by adsorption chromatography on aluminium oxide using hexane and benzene as eluants. This technique was suitable for the separation of feather lipids of land birds. The lipids of water birds formed difficult emulsions.

Saponification. This was effected by refluxing in 3 p.c. methanolic potassium hydroxide for four hours.

Esterification. The acetate was prepared by boiling with acetic anhydride, or by treatment at 100° C. in the presence of pyridine. The benzoate and the dinitrobenzoate were prepared by the usual method using pyridine as solvent.

Reference specimens.

Ergostanyl acetate was prepared by a two-stage catalytic hydrogenation of ergosteryl acetate using Adam's platinum oxide (Reindel, Walter and Rauch, 1927). The first hydrogenation in acetic acid resulted in $\Delta^{8(14)}$-ergostenyl acetate, which was isomerised with hydrogen chloride resulting in Δ^{14}-ergostenyl acetate. A second hydrogenation in ether yielded ergostanyl acetate, m.p. 143° C.

A direct hydrogenation of all three unsaturated linkages of ergosterol was effected by the method of Heilbron and Sexton (1929), by hydrogenation of a solution of ergosterol in acetic acid with palladium-charcoal. The reaction mixture was shaken for 8 hours in an isomantle kept at 80° C. The resulting ergostanyl acetate was hydrolysed, and the ergostanol purified by passing a hexane solution through an aluminium oxide column and subsequent elution with benzene.

Plumin.

The solids of the hexane fraction, which were sparingly soluble in methanol, were submitted to repeated chromatography on columns of aluminium oxide prepared in hexane and elution with hexane. The material so obtained was recrystallised from acetone and ethanol to give colourless crystals of plumin (I), m.p. 92° C., $[\alpha]_D + 7{\cdot}5°$ C. (*c*, 1·8 in chloroform), $\nu_{max}^{CCl_4}$ 2915, 1737, 1462, 1180, 725 cm^{-1}. [Found (after drying at 50°/0·01 mm. for 4 hours): C, 82·58; H, 12·59. $C_{46}H_{84}O_2$ requires C, 82·56; H, 12·65.] The infra red absorption spectrum suggested that plumin is an ester. It gave a positive Liebermann-Burchardt reaction but did not yield an insoluble digitonide with a 1 p.c. ethanolic solution of digitonin.

On saponification plumin yielded stearic acid, m.p. 67° C. after crystallisation from ethanol, [Found: C, 76·21; H, 12·62; M, 282·6. Calc. for $C_{18}H_{36}O_2$: C, 75·99; H, 12·76; M (monobasic), 284·5] whose infra red absorption spectrum was identical with that of an authentic specimen, and ergostanol, m.p. 140° C., $[\alpha]_D + 14°$ (*c*, 1·0 in chloroform) after recrystallisation from methanol, [Found: C, 83·41; H, 12·37. Calc. for $C_{28}H_{50}O$: C, 83·57; H, 12·52], whose infra red absorption spectrum was identical with that reported for ergostanol (II, Fig. 1) by Dobriner *et al.* (1953), and with that of a specimen, m.p. 143° C., prepared by complete hydrogenation of ergosterol.

The acetate, which showed an infra red absorption spectrum identical with that of ergostanyl acetate, m.p. 145° C., $[\alpha]_D + 6°$, as prepared by us and as reported in the literature (Dobriner *et al.*, 1953), had, however, the markedly lower m.p. 109° C., $[\alpha]_D + 9{\cdot}5°$, probably a polymorphic form [Found: C, 81·06; H, 11·85. Calc. for $C_{30}H_{52}O_2$: C, 81·08; H, 11·79]. The benzoate, m.p. 156° C. (softens at 136° C. with blue thermochromism and gives a clear melt at 156° C.), $[\alpha]_D + 16°$ (*c*, 1·0 in chloroform), had an infra red spectrum identical with that of genuine ergostanyl benzoate m.p. 162° C., $[\alpha]_D + 13°$ (*c*, 1·2 in chloroform) [Found: C, 82·67; H, 10·92. Calc. for $C_{35}H_{54}O_2$: C, 82·95; H, 10·74]. The dinitrobenzoate had m.p. 185° C., $[\alpha]_D + 12°$ (*c*, 1·1 in chloroform) [Found: C, 70·19; H, 8·80; N, 4·85. Calc. for $C_{35}H_{52}O_2N_6$: C, 70·44; H, 8·78; N, 4·69].

In Table 2 data on (II) and its esters are summarised and compared with the published data for authentic ergostanol and corresponding esters.

TABLE 2.

Comparison of plumin steroid and authentic ergostanol.

Name	Plumin Steroid		Authentic Ergostanol	
	m.p. °C.	$[\alpha]_D$	m.p. °C.	$[\alpha]_D$
Steroid	140	+14·0	141–145	+16·0
Acetate	109	+ 9·5	145	+ 6·0
Benzoate	156	+16·0	162–165	+13·0*
Dinitrobenzoate	184	+12·0	202	+14·0

* Determination by authors; other figures for authentic compounds are from the literature.

The benzene eluate yielded a yellow extract which was subdivided into a small hexane fraction which was shown to consist of plumin and a large benzene eluate which was found to be identical with (II) as shown by analysis, melting point, infra red absorption and preparation of the identical acetate, m.p. 109° C.

The stearic acid isolated from the alcohol and acetic acid eluates was identified by infra red spectroscopy and mixed melting point with an authentic specimen.

RESULTS.

General composition.

The feathers were extracted with ether. After evaporating the ether some general properties were determined (Table 1) on the non-volatile material referred to as feather lipids or feather fat. It is noteworthy that neither squalene, a main constituent of human sebum (MacKenna, Wheatley and Wormall, 1950), nor octadecanol, the main constituent of preen gland secretion (Weitzel and Lennert, 1951), could be found in the extract of fowl or pigeon.

The feather lipids were then submitted to chromatography on alumina and four fractions were collected by elution with (a) hexane, (b) benzene, (c) ethanol, and (d) acetic acid.

Fowl feather lipids.

(a) The solids of the hexane eluate constituted the largest fraction of the feather lipids, and from roosters caponised with stilboestrol it made up 54 p.c.

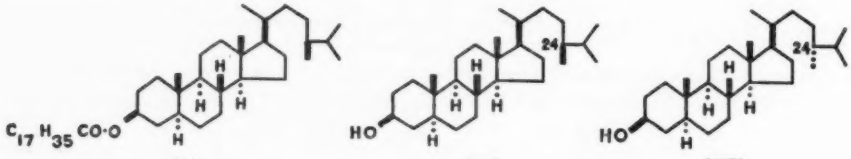
of the total ether soluble material. The feathers of normal chickens yielded about 45 p.c.

On saponification the purified solid of the hexane fraction yielded ergostanol (ergostan-3β-ol, II) and stearic acid. This ergostanol stearate was referred to as plumin (I) (Bolliger and Gross, 1957).

(b) The benzene eluate yielded about 10 p.c. of the total feather lipids, which were shown to be mainly free ergostanol (II).

(c) The ethanol eluate consisted of a small amount of an ill-defined mixture of substances, from which only stearic acid could be isolated.

(d) The acetic acid eluate consisted of a mixture of fatty acids, the main constituent being stearic acid.



Pigeon feather lipids.

The hexane eluate of pigeon feather lipids contained 25 p.c. of plumin identical with that obtained from fowl. The benzene eluate consisted mainly of ergostanol (6-10 p.c. yield), which was identical with (II) from fowl. Other fractions were not investigated.

Duck feather lipids.

The hexane eluate contained about 25 p.c. of plumin as well as large but varying amounts of an oil, and the benzene fraction consisted mainly of ergostanol (II).

Lipids from the feathers of geese and seagulls.

Plumin and ergostanol were obtained from the feathers of these birds in approximately similar amounts to those of the duck. An oil similar to that obtained from ducks was also present in the hexane fraction (a) of these feathers.

DISCUSSION.

The mammalian skin and its keratinous appendages are impregnated and covered by water soluble and lipid substances. There are two contributors to the lipids, the sebaceous glands and the by-products of the process of keratinisation, the ether soluble non-keratins.

For years investigators have been interested in separating these two groups of lipids, attempting on the one hand to obtain sebaceous material alone and on the other hand those lipids supplied by keratinisation, without much success.

Birds possess practically only one sebaceous gland, the preen gland and the lipids of this gland as obtained from the duck have been extensively examined (Weitzel and Lennert, 1951). In certain birds of prey, however, the preen gland is absent. In other land birds, such as fowl and pigeon, the gland is small and not well developed (Esther, 1938; Cater and Lawrie, 1950) and the typical preening activity comparable to that observed in water birds is not or only rarely observed. Any ether soluble organic substances derived from their feathers can therefore be assumed to be mainly by-products of keratinisation and not sebaceous secretion from the preen gland.

In contrast to land birds the preen gland of aquatic birds such as the duck is large and highly developed to produce considerable amounts of oily secretions, which are said to render the feathers impermeable to water. Consequently, feather lipids are contaminated by preen gland secretion of an oily nature and the total ether extractables are high in the duck (2 p.c.) compared with those of the castrated rooster (0·8 p.c.) which contain no oily substances.

However, in all species examined a steroid ester (plumin) was present and the scanty feather lipids of the castrated rooster yielded the largest percentage.

Plumin and plumin steroid.

The acid portion of plumin was stearic acid and according to elementary analyses and infra red absorption, the steroid moiety, which also occurred as such in the feather lipids (fraction b), seemed to be ergostanol. However, there were some differences in melting point, specific rotation and Liebermann-Burchardt reaction between (II) and some of its esters and the corresponding compounds of authentic ergostanol (Table 2). The difference in melting point was particularly marked in the acetate, m.p. 145° C. for the synthetic product obtained from ergosterol, but 109° C. for the steroid obtained from feather fat. These discrepancies may be due to polymorphism, stereoisomerism, or contamination by an isomer, e.g., the $C_{(24)}$-epimeride campestanol (III).

In this connection it may be mentioned that it has been suggested that stellastanol, the hydrogenation product of the starfish sterols, is not a uniform compound but rather campestanol containing a certain amount of its $C_{(24)}$-epimeride, ergostanol (Ruigh, 1945). Similarly, it has been suggested that campestanol prepared from the sterol of the sponge *Chalina*, chalinasterol, is contaminated by ergostanol (Bergmann, Schedl and Low, 1945). It appears, therefore, that practically irremedial contamination of campestanol by ergostanol and *vice versa* may be expected. This may explain some of the discrepancies between authentic ergostanol and the ergostanol obtained from feathers.

Lipids obtained by washing whole mammals or their fur in organic solvents inevitably consist of a mixture of non-keratins and sebum, but these products have frequently been considered as "sebum". Thus it has been mentioned by Wheatley and James (1957) that a steroid, cholest-7-en-ol (lathosterol) is characteristic of the sebum of rodents. Plumin and plumin steroid seem to be

characteristic of the plumage of birds, but it is suggested that they are not derived from sebum. Firstly, they occur in comparable amounts in the non-preening fowl and in typical preening birds. Moreover, octadecyl alcohol which constitutes about half of the preen gland content of the duck and fowl could not be found in the feather extracts of the fowl. In addition, the presence in preen glands of only about 1·4 p.c. of steroid in the form of cholesterol which, however, could not be demonstrated histologically (Weitzel and Lennert, 1951; Lennert and Weitzel, 1951) throws further doubt on the preen gland origin of feather lipids in fowl and pigeon which contain 30-40 p.c. of steroids. It is suggested, therefore, that in birds the steroid fraction originates largely from the process of keratinisation. In mammals a similar origin of steroids of the integument may be considered.

SUMMARY.

Extraction of birds' feathers with organic solvents yields from 0·8 to 2·5 p.c. of lipid material. Fractionation of these feather lipids resulted in a high proportion of a steroid stearate (plumin), its free steroid moiety (plumin steroid), stearic acid and other, unidentified fatty acids.

The plumin steroid and some of its esters have been examined and compared with related compounds, and the steroid is considered to be ergostanol.

It is suggested that the feather lipids are derived from the keratinisation process and are not sebaceous secretions with the exception of the feather lipids of water birds such as duck, goose and seagull which contain additional lower melting substances probably derived from the preen (sebaceous) gland.

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ZINC TOXICITY IN THE RAT AND ITS INTERRELATION WITH COPPER

by D. R. GRANT-FROST AND E. J. UNDERWOOD

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Most of the early work on zinc toxicity in animals is difficult to evaluate since neither the zinc intake nor the detailed nature of the rest of the diet is given. The first quantitative studies are those of Heller and Burke (1927) and Sutton and Nelson (1938), who added zinc to otherwise satisfactory diets in concentrations ranging from 0·1 p.c. to 1·0 p.c. and found that the effects on rats ranged from no effect at the 0·1 p.c. level to severe anaemia, markedly subnormal growth, reproductive failure and some mortality at the 1 p.c. level. Stimulated by these findings, Smith and Larson (1946) carried out more critical studies which revealed that the addition of zinc to an otherwise adequate diet for rats induces at least two distinct effects—a microcytic, hypochromic anaemia that can at least partially be overcome by an additional supplement of copper salts, and subnormal growth that can be partially prevented by an additional supplement of liver extract. The copper did not affect the growth-rate, nor did the liver extract affect the anaemia. Van Reen (1953) confirmed and extended these findings. He found that 0·5 to 0·7 p.c. zinc as the carbonate, added to a diet containing 8 p.p.m. copper, reduced growth, food consumption and haemoglobin levels in young rats and produced a marked reduction in liver catalase and cytochrome oxidase activities. Feeding additional copper at the rate of 0·2 mg. or 0·4 mg. Cu daily as the sulphate maintained the liver catalase and cytochrome oxidase activities of the zinc-fed rats at normal levels but had no effect on the growth of the animals.

It is apparent that these workers have left certain important questions unanswered. For instance, it is not known whether the subnormal growth which occurs in zinc toxicity is due wholly or in part to reduced food consumption or whether the anaemia is caused by a depression in copper assimilation in the animals, by increased excretion or by an antagonistic effect of the zinc on the copper within the tissues. The present experiment is designed to throw some light on these problems.

MATERIALS AND METHODS.

An experiment of simple factorial design was carried out, in which 5 groups of 6 rats in each group were fed as follows:

Group A: Basal diet; Group B: Basal diet + copper; Group C: Basal diet + zinc; Group D: Basal diet + copper + zinc; Group E: Basal diet (food consumption restricted to that of Group C).

Wistar hooded male rats, 5-6 weeks of age at the commencement of the experiment, were selected from six litters and distributed so that as far as possible litter mates occurred in different groups and the mean weights of the groups were similar. Each animal was allotted an individual metabolism cage at random and each group was given a position in the housing room at random. The cages of the first four groups were constructed of galvanised iron, coated with hard plastic enamel to exclude all contact with zinc and fitted with aluminium funnels which led to glass collection vessels allowing the separate collection of urine and faeces. The grid floor of each cage was made of chrome-plated steel. Group E was housed in chrome-plated wire mesh cages raised to avoid access to faeces.

The basal diet consisted of a mixture of 80 p.c. whole crushed wheat and 20 p.c. whole dried milk and contained an average of 2·1 p.p.m. Cu. This diet was fed *ad libitum*, but a daily record of feed consumption was kept. Copper-free water was available at all times. Zinc was added at a level of 0·5 p.c. Zn as the oxide and mixed into the feed of Groups C and D. This level was chosen because it was expected that it would produce anaemia and subnormal growth without heavy mortality. Supplementary copper was given as copper sulphate to each animal of Groups B and D each day at the rate of 0·4 mg. Cu daily. This was administered in 5 ml. of fresh milk in small glass jars and was offered to the animals *before* being given their dry feed. It is possible that this routine could have an effect on the results of the experiment as will be explained later. Groups A, C and E were given 5 ml. of milk only each morning in the same manner.

The rats were weighed three times each week and haemoglobin determinations made at the beginning, every two weeks and at the end of the six weeks' experiment. Feed residues were weighed daily and the food consumption of the animals of Group E maintained each day at that of the zinc group (Group C) on the previous day. Some spillage of milk and of faeces and contamination of faeces by urine and urine by faeces occurred so that complete copper balances, as originally intended, were not attempted.

At the end of six weeks' experimental regime all animals were anaesthetised with ether, their body cavities opened and blood removed for analysis by heart puncture. Liver and kidneys were removed, weighed, dried at 105° C. and the dry weight determined. The alimentary canal was removed and discarded and the remaining part of the body dried and ground. With blood, liver and kidney the whole sample was analysed for copper, but the copper and the fat contents of the body were determined on suitable aliquots. All copper values were obtained by a modification of the method of Eden and Green (1940).

RESULTS.

Growth and food consumption. The average live-weights of the rats given the different treatments at the beginning and end of the six weeks' experimental period are presented in Table 1, together with the food consumption data.

The results for all five groups were submitted to an analysis of variance and, in addition, Groups C and E were compared by the "t" test. A highly significant variation between groups was obtained both with the weight gains and the food consumption data. In neither case was the copper-zinc interaction significant, but the effect of zinc on growth and food consumption was highly significant at the 0·1 p.c. level. Variation due to copper was significant at the 5 p.c. level

TABLE 1.

The effect of copper and zinc on the growth and food consumption of rats.

(Wts. in gm.)

Group	Treatment	Av. wt. at 0 weeks	Av. wt. at 6 weeks	Av. wt. increase	Av. food consumption per week	Food eaten per gm. wt. gain
A	Basal	85	202	117	87	4·5
B	Basal+Cu	84	208	124	88	4·3
C	Basal+Zn	80	116	36	54	8·9
D	Basal+Cu+Zn	78	135	57	59	6·3
E	Basal (R)	79	111	32	54	10·0

in the case of the weight gains, but not in the case of the food consumption. There is no significant difference between Groups C and E.

Haemoglobin values. The mean haemoglobin values of the rats in relation to treatment at 0, 2 and 6 weeks are given in Table 2.

Differences between groups are highly significant at 2 weeks and at 6 weeks, but at neither time is the copper-zinc interaction significant. The effects of copper and of zinc are significant at both times and the difference between Groups C and E at 6 weeks, when compared by the "t" test, is also highly significant ($p < 0{\cdot}001$).

Copper concentrations. The average concentrations of copper in the blood, liver, kidney and body of the rats following six weeks of each treatment are given in Table 3.

With each tissue, the copper, zinc and copper-zinc effects are all highly significant ($p < 0{\cdot}001$), while these effects are significant ($p < 0{\cdot}01$) for the whole body. The treatment differences between Groups C and E are also all highly significant.

TABLE 2.

The effect of copper and zinc on the haemoglobin levels of rats.

(gm. Hb per 100 ml. blood)

Group	Av. value at 0 weeks	Av. value at 2 weeks	Av. value at 6 weeks	Av. change in 2 weeks	Av. change in 6 weeks
A	11·7	12·6	13·7	+ 0·9	+ 2·0
B	11·8	14·2	15·4	+ 1·5	+ 3·6
C	12·3	10.3	7·3	− 2·0	− 5·0
D	12·6	12·0	11·3	− 0·6	− 1·3
E	—	—	14·5	—	—

TABLE 3.

The effect of copper and zinc on tissue copper concentrations.

(p.p.m. Cu on dry basis)

Group	Blood	Liver	Kidney	Body	Body (fat-free)
A	1·03	12·5	21·6	3·7	6·0
B	1·23	15·0	23·4	3·7	5·8
C	0·25	6·7	10·0	2·3	2·9
D	1·34	16·0	22·2	3·8	5·5
E	1·05	15·0	19·8	4·4	6·3

Total copper values. The average total copper contents of the liver, kidneys and whole bodies (less blood, liver, kidneys and intestinal contents) of the rats in each group at the end of the treatment period are set out in Table 4.

TABLE 4.

The effect of copper and zinc on total tissue copper contents.

(Amounts in μg. Cu)

Group	Liver	Kidney	Body
A	164·7	48·6	1417·1
B	191·2	52·9	1443·7
C	43·3	15·5	398·8
D	121·3	36·2	857·5
E	100·7	29·8	886·7

As with the copper concentrations, the differences between groups are highly significant ($p < 0·001$); the copper and zinc effects are similarly highly significant; and the copper-zinc interaction significant ($p < 0·01$). Application of the "t" test to Groups C and E showed that the treatment differences are highly significant for liver and significant for body and kidney values.

Body fat values. The mean percentage body fat for the various treatments were as follows: Group A, 38·6; B, 35·7; C, 21·6; D, 30·3; E. 30·3.

The copper effect is non-significant, the zinc effect highly significant and the copper-zinc interaction significant ($p < 0·05$). An analysis of variance on Groups C and E showed the difference to be significant ($p < 0·05$) but when the "t" test was employed the difference appears non-significant.

DISCUSSION.

The earliest and most obvious sign of zinc toxicity was found to be a marked reduction in food consumption, accompanied by a depression in growth rate. Food intake was reduced in the zinc-fed animals from the first day of treatment, pointing strongly to unpalatability as the main causal factor. The total reduction in food consumption in Group C was 38 p.c., compared with a 25 p.c. re-

duction in the experiments of Van Reen (1953) where similar levels of zinc, as zinc carbonate, were used. This suggests that zinc oxide is more unpalatable, or more toxic, or both, than zinc carbonate. From a comparison of the weight gains and food consumption figures for the zinc-toxic animals of Group C and those of Group E, in which the food intake was maintained at the same level as that of Group C, it would appear that the effect of zinc on growth can be wholly accounted for by the lowering of food consumption and of food utilisation (Table 1).

It should be noted, however, that the addition of copper to the zinc-fed animals (Group D) had a small but significant effect in improving growth without any corresponding effect on food consumption, and the rats of this Group had significantly lower haemoglobin values than those of Group E. Lowered food consumption could not, therefore, of itself, be responsible for the anaemia and the possibility exists that the zinc-induced anaemia contributed to the poor growth. It seems that the major cause of the poor growth in zinc toxicity is a reduction in food intake, due in this case to unpalatability of the ration, but that metabolic effects of zinc contribute also to this effect.

According to Gallagher (1957) anaemia is a relatively late sign of simple copper deficiency in the rat and this anaemia is consequent upon a severe depletion of cytochrome oxidase activity following depletion of the animal's copper reserves. In our zinc-toxic animals (Group C), also copper deficient, a highly significant reduction in haemoglobin concentration occurred within 2 weeks of feeding the zinc and could have occurred earlier. This difference between the two types of copper deficiencies again suggests that zinc, besides reducing copper absorption, clearly indicated by the low blood and tissue copper concentrations and amounts (Tables 3 and 4), also had an effect at the cellular level. In other words, the zinc-toxic animal is unable to utilise its copper reserves because of an antagonistic effect of the zinc absorbed from the diet. There is no direct experimental support for this hypothesis, but additional evidence in this direction can be adduced from a comparison of Groups A and D. The haemoglobin levels for the animals of Group D (copper + zinc) are significantly lower than those of the controls at the end of the experiment, although there is certainly no lack of copper in their blood and tissues. Either, therefore, the absorbed zinc is reducing the availability of the tissue copper, as just suggested, or a deficiency of some other dietary essential affecting haematopoiesis is occurring as a consequence of the high zinc intakes.

The outstanding features of the copper values are the very low levels in the animals receiving the zinc supplement alone and the complete maintenance of these values at normal levels by the further addition of 0·4 mg. Cu per day. The former can mean either that the zinc has reduced copper absorption, promoted excretion or both. In the absence of copper balance studies, which were planned but rejected owing to spillage and faecal and urine contamination,

conclusive evidence for either one of these cannot be presented. The normal copper contents of the animals receiving the copper supplement in addition to the zinc might be taken as support for the contention that the main effect of zinc is to increase excretion of absorbed copper, but the timing and form of the copper supplementation may be of considerable significance. In our experiment, and indeed in all previous experiments involving zinc and copper feeding, the copper was presented in a small quantity of milk and was consumed prior to the main ration containing the zinc. Copper, therefore, entered the alimentary canal at a time when it was relatively free from zinc and could have been absorbed before any effect of zinc on copper availability in this site was fully exerted. In the future, it would appear essential to administer the copper and zinc together. In spite of this, there is no doubt that zinc, at the level used in this experiment, profoundly reduces copper *retention* and induces a marked copper deficiency within the animal. The blood copper values are, in fact, so low that normal haematopoiesis could not take place.

The observation of Sadasivan (1951) that excess zinc in the diet reduces the liver fat content of rats finds support from our findings. The fat content of the bodies of the zinc-fed animals of Group D was significantly lower than that of any other group, including Group E which received the same amount of food but no zinc. Lowered food consumption is clearly therefore not the reason for this effect. It can be argued, however, that the reduction in body fat is not due, as Sadasivan states, to a lipotropic action of zinc by depressing lipase activity, but to the copper deficiency. Gallagher (1957) has shown that phospholipid synthesis is depressed in copper deficiency and concludes that it is the attachment of fatty acids of glycerophosphate which is affected. This process needs studying under conditions of zinc toxicity.

SUMMARY.

An experiment was carried out to study the effect on growing rats of the addition of 0·5 p.c. of dietary zinc, as zinc oxide, and of the relation of supplemental copper to zinc toxicity.

The zinc was found markedly to reduce the growth, food consumption, haemoglobin levels, copper retention and body fat content of the animals.

The addition of 0·4 mg. Cu daily to the diet of the zinc-fed rats was found to have no effect on food consumption, to improve growth slightly but significantly, to maintain the copper contents of blood and tissues at normal levels and to afford considerable protection against the anaemia.

It was concluded that:

(a) The depressing effect of zinc on growth is caused largely but not entirely by reduced food consumption, probably due to the unpalatability of the zinc-containing diet; (b) that anaemia is caused by a zinc-induced copper deficiency in the animals; and (c) the zinc not only profoundly reduces the copper

concentrations in the blood and tissues but probably also antagonises absorbed copper at the cellular level.

The importance of the nature and timing of the zinc and copper supplements to a proper understanding of zinc-copper interrelations is stressed.

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BEMEGRIDE ANALEPSIS TO DEEP HYPNOSIS INDUCED BY A VARIETY OF HYPNOTICS

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(*Accepted for publication 1st April, 1958.*)

The antagonism of Bemegride (I) (β-methyl-β-ethylglutarimide, Megimide, NP13) to moderately deep hypnosis in mice (sleeping time 60-80 minutes), induced by hypnotics both structurally related and unrelated to it has been indicated previously (Shaw, Simon, Cass, Shulman, Anstee and Nelson, 1954; Shulman, 1956; Somers, 1956; Frey, Hushahn and Soehring, 1956; Kimura and Richards, 1957; Shulman and Laycock, 1957a; and Shulman and Laycock, 1957b and c). However, apart from the work of Kimura and Richards (1957), no systematic laboratory study appears to have been undertaken to assess the effectiveness of Bemegride analepsis in the case of deep hypnotic coma induced by these drugs.

This aspect has been investigated employing a method in which Bemegride is gradually titrated against each hypnotic in a manner resembling that advocated for the management of clinical cases of comparably deep coma (Shulman, Shaw, Cass and Whyte, 1955). This method was used in an attempt to reduce toxicity and lethality which could result from the acute administration of the large doses of Bemegride required for effective antagonism to be exhibited.

The hypnotics used in this study are: α-bromo-α-ethylbutyrylurea (II) (Carbromal), chloral hydrate (III), 5-ethyl-6-phenyl-2:4-diketo-metathiazane[2] (IV) (Dolitrone) (Lundy, 1955), 5:5-diethyl-2:4-diketothiazolidine[3] (V) (Erlenmeyer, 1938), α-ethyl-α-phenylglutarimide[4] (VI) (Glutethimide, Doriden) (Gross, Tripod and Meier, 1955), α-methyl-α-phenylglutarimide[5] (VII) (Marshall and Vallance, 1955), β-methyl-β-*n*-amylglutarimide[6] (VIII) (Somers, 1956), α-methyl-α-phenylsuccinimide[7] (IX) (Chen, Portman, Ensor and Bratton, 1951), N-methyl-α-methyl-α-phenylsuccinimide[5] (X) (Chen, Portman, Ensor and Bratton, 1951; Marshall and Vallance, 1954), 3:3-diethyl-5-methyl-2:4-diketopiperidine[3] (XI) (Methypyrlone, Noludar) (Pellmont, Studer and Jürgens, 1955),

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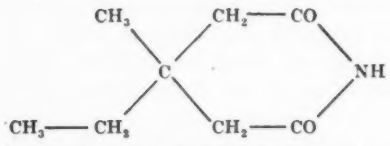
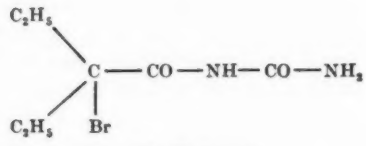
[2] Wm. S. Merrell & Co., U.S.A. [3] F. Hoffman-La Roche, Basel. [4] C.I.B.A. (Basel). [5] British Schering Research Laboratories Ltd. (England). [6] Nicholas Pty. Ltd. (Australia). [7] Parke, Davis & Co. Ltd. (U.S.A.). [8] Abbott Research Laboratories (U.S.A.). [9] Chas. Pfizer & Co. Inc. (U.S.A.).

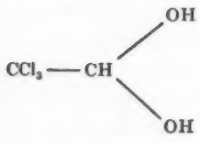
3-methylpentyn-3-ol[5] (XII) (Methylpentynol, Oblivon) (Margolin, Perlman, Villani and McGavack, 1951; Marshall and Vallance, 1954), paraldehyde (XIII), pentobarbitone (XIV), phenyl-α,α-diethylacetamide[5] (XV) (Chapman, McCrea, Marshall and Sheahan, 1957), 3:3-diethyl-2:4-diketo-tetrahydropyridine[3] (XVI) (Persedon) (Pellmont, Studer and Jürgens, 1955), β-chlorvinyl-ethyl-ethynyl-carbinol[8] (XVII) (Placidyl) (P'an, Kodet, Gardocki, McLamore and Bavley, 1955; Kimura and Richards, 1957), urethane (XVIII) and 21-hydroxypregnanedione sodium succinate[9] (XIX) (Viadril) (P'an, Gardocki, Hutcheon, Rudel, Kodet and Laubach, 1955).



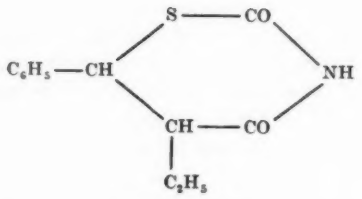
I. β-methyl-β-ethylglutarimide (Bemegride, Megimide, NP13)



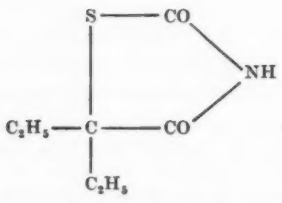
II. Carbromal (α-bromo-α-ethylbutyrylurea)



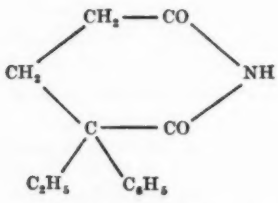
III. Chloral hydrate



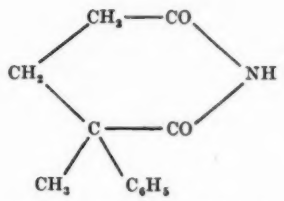
IV. Dolitrone (5-ethyl-6-phenyl-2:4-diketometathiazane)



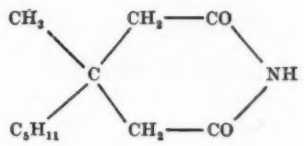
V. 5:5-diethyl-2:4-diketothiazolidine



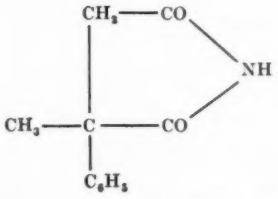
VI. Doriden (α-ethyl-α-phenylglutarimide)



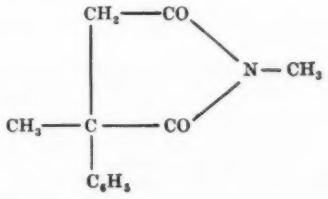
VII. α-methyl-α-phenylglutarimide



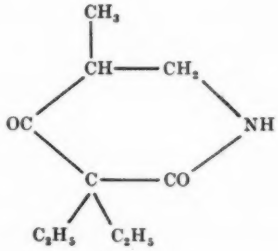
VIII. β-methyl-β-amylglutarimide



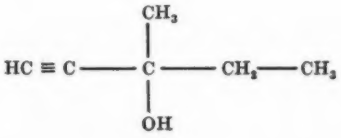
IX. α-methyl-α-phenylsuccinimide



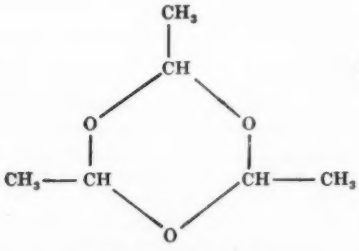
X. N-methyl-α-methyl-α-phenylsuccinimide



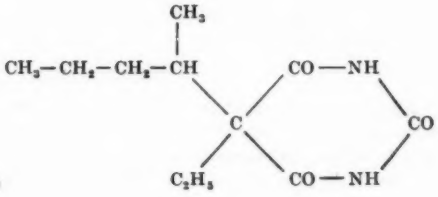
XI. Noludar
(3:3-diethyl-5-methyl-2:4-diketopiperidine)



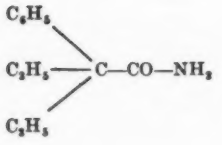
XII. Oblivon
(3-methyl-pentyn-3-ol)



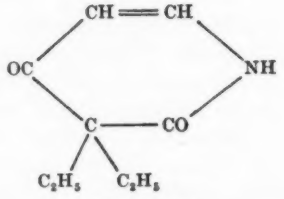
XIII. Paraldehyde



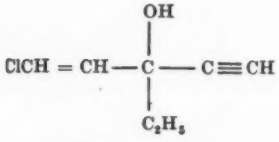
XIV. Pentobarbitone



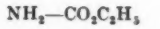
XV. Phenyl-α,α-diethylacetamide



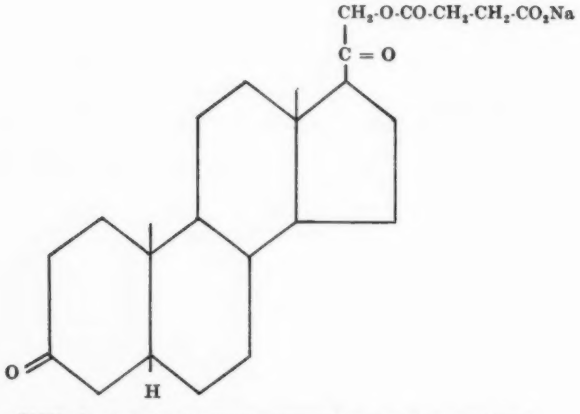
XVI. Persedon
(3:3-diethyl-2:4-diketo-tetrahydropyridine)



XVII. Placidyl
(β-chlorvinyl-ethyl-ethynylcarbinol)



XVIII. Urethane



XIX. Viadril (21-hydroxypregnanedione sodium succinate)

EXPERIMENTAL DETAILS.

Albino mice of mixed sex and weighing between 20 and 30 gm. were divided by random selection into test groups of 15. The analeptic activity of Bemegride was demonstrated by its ability to reduce the sleeping time (and often lethality) for mice given a dose of hypnotic so chosen as to produce deep hypnosis (sleeping time 2 to 12 hours, minimal or no response to pain, marked depression or absence of corneal reflex and frequent cyanosis often accompanied by death). No attempt was made to produce hypnosis of quantitatively equivalent depth with each hypnotic. The sleeping time was considered as the time interval elapsing

between the loss and maintained recovery of the righting reflex. All drugs were administered intraperitoneally either in physiological saline solution (Bemegride, chloral hydrate, Dolitrone, Noludar, pentobarbitone sodium, Persedon, urethane and Viadril) or, when necessary, in a 1 p.c. tragacanth suspension (Carbromal, Doriden, 5:5-diethyl-2:4-diketothiazolidine, β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide, α-methyl-α-phenylsuccinimide, N-methyl-α-methyl-α-phenylsuccinimide, Oblivon, paraldehyde, phenyl-diethylacetamide and Placidyl).

An initial injection of Bemegride (60 mg/Kg, $2 \times LD_{50}$, Kimura and Richards, 1957) was administered to the treated group simultaneously with each hypnotic to reduce the possibility of acute hypnotic death. This was followed by up to 6 injections of Bemegride (30 mg/Kg) at 7 minute intervals, the number of injections being determined by the prior awakening or death of the animals. The overall volume of fluid administered to the control (saline) and treated groups was not greater than 2·2 ml.

The results of these experiments are seen in Table 1. Signs of toxicity both during and after Bemegride administration were recorded.

A more detailed study was made of Bemegride activity in reversing deep hypnosis induced by hypnotics of very close structural similarity (β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide and α-methyl-α-phenylsuccinimide). This followed an observation that a dose of these hypnotics slightly higher than that indicated in Table 1 frequently resulted in less pronounced antagonism and increased lethality. Comparative experiments, employing the same technique, were carried out at 4 or 6 dose levels with these drugs and with two other structurally related hypnotics (Noludar and pentobarbitone) and two structurally unrelated hypnotics (chloral hydrate and Viadril) to determine the approximate hypnotic concentration where Bemegride ceased to be a safe and effective analeptic. The results of these experiments are seen in Table 2. The action of Bemegride in reversing hypnotic-induced depression of respiration, corneal reflex and response to pain and in producing signs of toxicity was also noted each 7 minutes following the administration of the analeptic.

RESULTS.

Table 1.

Bemegride significantly reduced the sleeping time ($p \leqslant 0{\cdot}01$ to $0{\cdot}001$) of mice deeply hypnotized with a large variety of structurally related and unrelated hypnotics.

Bemegride toxicity. Mild and transient signs of toxicity (tremors, Straub tail) were observed in Bemegride-treated animals having woken from hypnosis due to Dolitrone, 5:5-diethyl-2:4-diketothiazolidine, Noludar and phenyl-diethylacetamide, whereas more marked and prolonged signs of toxicity (tremors, Straub tail, convulsions and death) were evident in mice which had recovered from hypnosis induced by glutarimides and succinimides (see also results in Table 2).

The effect of Bemegride at a number of dose levels of β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide, α-methyl-α-phenylsuccinimide, pentobarbitone, Noludar, chloral hydrate and Viadril is seen in Table 2. A small increase in the dose of β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide and α-methyl-α-phenylsuccinimide resulted in a progressive increase in

TABLE 1.

Hypnotic and dose (mg/Kg)	Average dose Bemegride given to treated group (mg/Kg)	Number of deaths (D) and mean sleeping time of survivors ($\bar{X}$)		Combined standard error	"t" test treated group vs. control group
		Control group	Treated grp.		
Carbromal (500)	214	D_1 (317) $\bar{X}_{14}$ (290)	D_3 (65)+ $\bar{X}_{12}$ (102)++	21·54	8·69***
Chloral hydrate (750)	214	D_5 (102) $\bar{X}_{10}$ (194)	D_3 (30) $\bar{X}_{13}$ (69)	13·05	9·57***
Dolitrone (500)	240	D_{13} (112) $\bar{X}_2$ (391)	$\bar{X}_{15}$ (167)	—	—
5:5-diethyl-2:4-diketothiazolidine (500)	240	D_5 (96) $\bar{X}_{10}$ (145)	D_2 (49) $\bar{X}_{13}$ (88)	14·69	3·85***
β-methyl-β-*n*-amylglutarimide (470)	196	D_3 (69) $\bar{X}_{12}$ (146)	D_6 (41) $\bar{X}_9$ (86)	9·20	6·55***
α-methyl-α- phenylglutarimide (225)	116	D_6 (83) $\bar{X}_9$ (130)	D_1 (54) $\bar{X}_{14}$ (19)	15·53	7·15***
Doriden (200)	240	D_7 (130) $\bar{X}_8$ (274)	$\bar{X}_{15}$ (169)	11·15	9·42***
α-methyl-α-phenylsuccinimide (250)	133	D_7 (118) $\bar{X}_8$ (120)	D_8 (34) $\bar{X}_7$ (22)	21·26	4·60***
N-methyl-α-methyl-α-phenylsuccinimide (450)	160	D_2 (32) $\bar{X}_{13}$ (328)	$\bar{X}_{15}$ (55)	17·00	16·09***
Noludar (400)	230	D_7 (171) $\bar{X}_8$ (367)	D_4 (77) $\bar{X}_{11}$ (145)	47·01	4·72***
Oblivon (1000)	132	D_2 (347) $\bar{X}_{13}$ (569)	$\bar{X}_{15}$ (25)	25·27	22·71***
Paraldehyde (2000)	60	D_7 (8) $\bar{X}_8$ (139)	$\bar{X}_{15}$ (6)	11·69	11·36***
Pentobarbitone (90)	234	D_1 (21) $\bar{X}_{14}$ (127)	$\bar{X}_{15}$ (74)	16·46	3·21**
Persedon (500)	240	D_1 (276) $\bar{X}_{14}$ (305)	D_3 (76) $\bar{X}_{12}$ (143)	15·36	10·53***
Phenyl-diethyl-acetamide (220)	218	D_3 (206) $\bar{X}_{12}$ (289)	$\bar{X}_{15}$ (69)	17·92	12·29***
Placidyl (250)	116	D_{10} (128) $\bar{X}_5$ (167)	$\bar{X}_{15}$ (22)	16·76	8·65***
Urethane (1750)	194	D_8 (198) $\bar{X}_7$ (415)	D_{15} (46)	—	—
Viadril (200)	240	D_{11} (105) $\bar{X}_4$ (171)	$\bar{X}_{15}$ (77)	18·04	5·20***

\+ D_3 (65) = 3 deaths, Mean death time was 65 minutes.
++ $\bar{X}_{12}$ (102) = 12 survivors, Mean sleeping time was 102 minutes.
** = highly significant ($p < 0·01$)
*** = extremely significant ($p < 0·001$)

TABLE 2.

Hypnotic and dose (mg/Kg)	Average dose Bemegride given to treated group (mg/Kg)	Number of deaths (D) and mean sleeping time of survivors ($\bar{X}$)		Combined standard error	"t" test treated group vs. control group
		Control group	Treated grp.		
β-methyl-β-*n*-amyl-glutarimide (310)	162	$\bar{X}_{15}$ (85)	$\bar{X}_{15}$ (29)	8·99	6·21***
(350)	212		D_3 (52)+ $\bar{X}_{12}$ (44)++	12·15	3·80***
(390)	214	D_1 (161) $\bar{X}_{14}$ (103)	D_4 (64) $\bar{X}_{11}$ (51)	11·03	4·72***
(430)	220	D_1 (45) $\bar{X}_{14}$ (125)	D_8 (38) $\bar{X}_7$ (78)	15·51	3·02**
(470)	196	D_3 (69) $\bar{X}_{13}$ (146)	D_6 (41) $\bar{X}_9$ (86)	9·20	6·55***
(510)	168	D_4 (43) $\bar{X}_{11}$ (185)	D_{13} (19) $\bar{X}_2$ (165)	25·28	0·78 N.S.
α-methyl-α-phenyl-glutarimide (200)	68	D_5 (133) $\bar{X}_{10}$ (177)	$\bar{X}_{15}$ (4)	19·19	8·99***
(225)	116	D_6 (83) $\bar{X}_9$ (130)	D_1 (54) $\bar{X}_{14}$ (19)	15·53	7·15***
(250)	224	D_{10} (236) $\bar{X}_5$ (327)	D_{13} (57) $\bar{X}_2$ (168)	—	—
(275)	236	D_{15} (134)	D_{15} (73)	—	—
α-methyl-α-phenyl-succinimide (200)	60	D_1 (122) $\bar{X}_{14}$ (55)	D_1 (46) $\bar{X}_{14}$ (5)	7·75	6·43***
(225)	90	$\bar{X}_{15}$ (82)	D_2 (30) $\bar{X}_{13}$ (12)	8·17	8·57***
(250)	133	D_7 (118) $\bar{X}_8$ (120)	D_8 (34) $\bar{X}_7$ (22)	21·26	4·60***
(275)	194	D_2 (113) $\bar{X}_{13}$ (113)	D_{14} (38) $\bar{X}_1$ (48)	—	—
Pentobarbitone (60)	94	$\bar{X}_{15}$ (54)	$\bar{X}_{15}$ (11)	7·13	6·03***
(90)	234	D_1 (21) $\bar{X}_{14}$ (127)	$\bar{X}_{15}$ (74)	16·46	3·21**
(120)	240	D_5 (14) $\bar{X}_{10}$ (178)	D_1 (42) $\bar{X}_{14}$ (138)	19·57	2·03*
(150)	240	D_{15} (10)	D_{11} (122) $\bar{X}_4$ (181)	—	—
Noludar (300)	166	D_2 (143) $\bar{X}_{13}$ (247)	D_1 (115) $\bar{X}_{14}$ (30)	30·16	7·17***
(400)	230	D_7 (171) $\bar{X}_8$ (367)	D_4 (77) $\bar{X}_{11}$ (145)	47·01	4·72***
(500)	226	D_{11} (264) $\bar{X}_4$ (334)	D_{10} (66) $\bar{X}_5$ (120)	44·03	4·86**
(600)	206	D_{14} (161) $\bar{X}_1$ (540)	D_{15} (45)	—	—
Chloral hydrate (600)	158	$\bar{X}_{15}$ (104)	$\bar{X}_{15}$ (31)	11·52	6·35***
(750)	214	D_5 (102) $\bar{X}_{10}$ (194)	D_3 (30) $\bar{X}_{12}$ (69)	13·05	9·57***
(900)	224	D_{11} (59) $\bar{X}_4$ (194)	D_8 (113) $\bar{X}_7$ (127)	29·87	2·24*
(1050)	208	D_{15} (40)	D_{10} (65) $\bar{X}_5$ (199)	—	—

TABLE 2 (*continued*)

Hypnotic and dose (mg/Kg)	Average dose Bemigride given to treated group (mg/Kg)	Number of deaths (D) and mean sleeping time of survivors ($\bar{X}$)		Combined standard error	"t" test treated group vs. control group
		Control group	Treated grp.		
Viadril (100)	144	$\bar{X}_{15}$ (62)	$\bar{X}_{15}$ (32)	4·63	6·57***
(200)	240	D_{11} (105) $\bar{X}_4$ (171)	$\bar{X}_{15}$ (77)	18·04	5·20***
(300)	226	D_{14} (59) $\bar{X}_1$ (147)	D_3 (107) $\bar{X}_{12}$ (118)	—	—
(400)	240	D_{14} (68) $\bar{X}_1$ (274)	D_9 (181) $\bar{X}_6$ (199)	—	—

$^{+}D_3$ (52) = 3 deaths, Mean death time was 52 minutes.
$^{++}\bar{X}_{12}$ (44) = 12 survivors, Mean sleeping time was 44 minutes.
N.S. = not significant.
* = significant ($p = 0{\cdot}05$)
** = highly significant ($p < 0{\cdot}01$)
*** = extremely significant ($p < 0{\cdot}001$).

lethality and a suggestive decrease in the efficiency of Bemegride analepsis. A larger percentage increase in the dose of the last four hypnotics still resulted, in most cases, in beneficial Bemegride antagonism and lower incidence of lethality. The exception was the group receiving an approximate LD_{100} of Noludar in which there was a decrease in the mean death time of the treated group.

Bemegride toxicity. Increased muscular tonus at the lower doses of Bemegride (60-120 mg/Kg) and extensor spasm at a higher Bemegride concentration (150-240 mg/Kg) was evident in all groups hypnotized with β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide and α-methyl-α-phenylsuccinimide (except when quick death resulted, as in the majority of animals receiving β-methyl-β-*n*-amylglutarimide 510 mg/Kg). These animals also showed minimal tremors, and sporadic bounding movements during the terminal stages of Bemegride administration and more marked tremors, convulsive movements and often death after waking. (Similar results were also obtained with α-ethyl-α-phenylglutarimide (Doriden) and N-methyl-α-methyl-α-phenylsuccinimide, Table 1.)

No signs of toxicity were seen during Bemegride treatment of groups hypnotized with chloral hydrate, pentobarbitone and Viadril, whereas mild tremors were seen during treatment and after recovery of Noludar-hypnotized mice. Mild tremors and a low incidence of death were observed in mice which had woken from Viadril hypnosis.

The effect of Bemegride on depressed respiration, corneal reflex and response to pain.

Respiratory depression. Bemegride generally decreased the rate and increased the amplitude of respiration in all groups receiving β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide, α-methyl-α-phenylsuccinimide and chloral hydrate. This was followed in the case of β-methyl-β-*n*-amylglutarimide (430, 470 and 510 mg/Kg groups) and α-methyl-α-phenylglutarimide and α-methyl-α-phenylsuccinimide (250 and 275 mg/Kg groups) by respiratory depression as the dose of Bemegride was increased. An increase in rate and amplitude of respiration generally resulted in the Noludar, pentobarbitone and Viadril treated groups. Further Bemegride administration produced respiratory depression in the groups receiving Noludar 500 and 600 mg/Kg.

Depression of corneal reflex. Bemegride was an effective antagonist at all dose levels of β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide α-methyl-α-phenylsuccinimide, Noludar and Viadril where depression of corneal reflex was evident. Antagonism was also obtained in groups hypnotized with pentobarbitone, 60, 90 and 120 mg/Kg, and chloral hydrate, 600 mg/Kg, whereas only slight antagonism was produced, even by high doses of Bemegride (210-240 mg/Kg), in groups receiving chloral hydrate 750-900 mg/Kg and no antagonism in groups receiving chloral hydrate, 1050 mg/Kg and pentobarbitone, 150 mg/Kg.

Analgesia. These results parallel those indicated for Bemegride reversal of depressed corneal reflex, except in the case of β-methyl-β-*n*-amylglutarimide 510 mg/Kg where no antagonism was obtained.

DISCUSSION.

The results demonstrate qualitatively Bemegride analepsis to deep hypnosis induced in mice by hypnotics of 15 chemically distinct types, some structurally related and others unrelated to Bemegride. A current quantitative investigation at moderate levels of hypnosis will be the subject of a separate paper (Shulman and Laycock—unpublished observations).

Bemegride's wide spectrum of activity should make it an extremely valuable analeptic, particularly when gradually administered so as to keep the animal (or patient) in the "safe state" (Shulman, Shaw, Cass and Whyte, 1955). The limited experimental evidence available at present does not justify more than a generalized comparison of the value of Bemegride with that of other analeptics in deep hypnosis. Kimura and Richards (1957), employing a single dose technique, have shown in mice, given a LD_{100} of various hypnotics, that Bemegride gives complete protection against pentobarbitone, 50 p.c. protection against chloral hydrate, but no protection against ethchlorvynol (Placidyl), whereas picrotoxin and pentylenetetrazole were effective only against pentobarbitone. Using a multiple injection technique, we found that Bemegride (in doses up to

240 mg/Kg) protects against highly lethal doses of chloral hydrate, ethchlorvynol and pentobarbitone, suggesting that the intrinsic toxicity of a high circulating concentration of analeptic may be an important limiting factor in the process of analepsis.

Other workers (Mousel and Essex, 1941; Eckenhoff, Schmidt, Dripps and Kety, 1949; Koppanyi and Fazekas, 1950; Nilsson, 1951; Cass, 1956; and Holten, 1956) have suggested that amphetamine, coramine, geastimol, α-lobeline, pentylenetetrazole, picrotoxin and succinate may often have limited therapeutic value, both clinically and experimentally, in deep hypnotic coma (particularly of barbiturate origin), whereas the clinical and experimental results of Bemegride therapy seem at present more promising (Shulman *et al.*, 1955; Cass, 1956; Clemmensen, 1956; Holten, 1956; von Planta and Klingler, 1956; and Shaw, 1957).

A study of the effect of serial Bemegride administration on depressed respiration, corneal reflex, response to pain and righting reflex has indicated that the manner of recovery followed the same integrated pattern now well recognized in clinical cases of barbiturate intoxication treated with Bemegride (Shulman *et al.*, 1955; Harris, 1955; Holten, 1956; von Planta and Klingler, 1956; and Wyke and Frayworth, 1957). The order of recovery of the above indices paralleled that indicated by Wyke and Frayworth for the anaesthetized patient, i.e., initial respiratory stimulation together with or followed by recovery of the corneal reflex and response to pain and later by recovery of the righting reflex. This may provide additional support for the suggestion that the process of Bemegride analepsis occurs in the reticulocortical activating system (von Planta and Klingler, 1956; Wyke, 1957; and Wyke and Frayworth, 1957). However, in some cases (β-methyl-β-*n*-amylglutarimide 470 and 510 mg/Kg, α-methyl-α-phenylglutarimide 275 mg/Kg, α-methyl-α-phenylsuccinimide 275 mg/Kg and Noludar 500 and 600 mg/Kg), respiratory depression occurred at a time when antagonism of the depressed corneal reflex and response to pain was maintained. This may indicate a differential saturation of the receptors subservient to these functions as the dose of Bemegride was increased.

Further information concerning the sites and mechanism of Bemegride analepsis in the central nervous system has been provided by unpublished experiments (Wright, Shulman and Laycock). This work has indicated a differential quantitative antagonism between equimolar concentrations of Bemegride and pentobarbitone titrated at different sites of neural integration using intact, decerebrate and spinal cats and rabbits.

A wide range in the micromolar/kilogram concentration required to produce deep hypnosis at a similar level was found for the series of hypnotics and in the corresponding concentrations of Bemegride required for significant analepsis. This observation is supported by the quantitative investigation of Bemegride activity at moderate levels of hypnosis induced by the same series of structurally diverse hypnotics (Shulman and Laycock—unpublished observations).

It is noted that as the dose of selected hypnotics (Table 2) is progressively increased Bemegride ceases to exert significant antagonism and indeed potentiates the lethality of β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide, α-methyl-α-phenylsuccinimide and Noludar. This occurs in the closely structurally related hypnotics (glutarimides and succinimides) with doses only slightly greater (8-10 p.c.) than those at which effective antagonism was still evident. This mechanism is reminiscent of the *in vitro* depressant action of excess substrate on an enzyme system and this possible interpretation, suggesting competitive antagonism, is at present under investigation.

It seems pertinent to stress the possible danger involved in too freely extrapolating our results in mice to the clinical treatment of deep hypnotic coma.

SUMMARY.

Bemegride analepsis has been demonstrated in mice deeply hypnotized by hypnotics of 15 chemically distinct types. Those structurally related to Bemegride are: Carbromal, Dolitrone, Doriden (Glutethimide), 5:5-diethyl-2:4-diketothiazolidine, β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide, α-methyl-α-phenylsuccinimide, N-methyl-α-methyl-α-phenylsuccinimide, Noludar (Methypyrlone), pentobarbitone and Persedon. Hypnotics structurally unrelated to Bemegride are chloral hydrate, Oblivon (Methylpentynol), paraldehyde, phenyl-α,α-diethylacetamide, Placidyl, urethane and Viadril.

In the case of selected hypnotics studied in more detail (chloral hydrate, β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide, α-methyl-α-phenylsuccinimide, Noludar, pentobaritone and Viadril), Bemegride administration produced the same integrated recovery seen in the comparable clinical treatment of deep barbiturate intoxication.

Evidence has been presented suggesting the possibility of competitive antagonism between Bemegride and the closely related hypnotics β-methyl-β-*n*-amylglutarimide, α-methyl-α-phenylglutarimide and α-methyl-α-phenylsuccinimide.

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THE NATURE OF THE ADSORBED ANTIBODY IN ACQUIRED HAEMOLYTIC ANAEMIA

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The importance of antibodies in the pathogenesis of acquired haemolytic anaemias is now universally recognised and their study has been greatly facilitated by the introduction of the anti-human globulin test (Coombs, Mourant and Race, 1945). The presence of an antibody adsorbed on to the red cells of many patients with acquired haemolytic anaemia may be demonstrated by this test and it was originally thought that the antibody concerned was a gamma globulin (Coombs and Mourant, 1947), but it was soon shown that this was not so in every instance (Dacie, 1951; Vaughan and Waller, 1954).

A method of immunological identification of the type of protein adsorbed on to the red cells has been described (Firkin and Blackburn, 1956), and this paper reports the results of the investigation of 8 patients with acquired haemolytic anaemia.

Methods.

All the patients studied had a positive direct Coombs test. Three had cold agglutinins and one of these also had a cryoglobulin which appeared to be unrelated to the cold agglutinins since cold agglutination still occurred after the extraction of the cryoglobulin. Three others had a warm type of agglutinin, but only one of these was actively haemolysing at the time of investigation. Two babies with haemolytic disease were also investigated.

Albumin, alpha 2, beta and gamma globulins were obtained by eluting with normal saline the appropriately identified zones of the paper electrophoretogram of normal serum (Wunderly, Gloor and Hassig, 1953). Antisera were prepared against isolated Bence Jones protein[3] (Collier and Jackson) and Fraction IV_7 and III (supplied by Commonwealth Serum Laboratories) in rabbits using the technique of Kabat and Mayer.

Warm agglutinins were eluted from carefully washed red cells by heating in saline to 56° C. for 5 minutes (Evans, Duane and Behrendt, 1947) and cold agglutinins were eluted by a slight modification of the method of Aubert and Brendemoen (1949) (Firkin and Blackburn, 1956). Ouchterlony's method of gel diffusion (Oudin, 1952) was then employed to identify the eluted proteins immunologically (Firkin and Blackburn, 1956).

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RESULTS.

The anti-IV_7 and anti-III rabbit sera contained anti-bodies to albumin, $alpha_2$, beta and gamma globulins. Fig. 1 illustrates the three types of reaction which may be obtained with Ouchterlony's technique of immunological identification. The central cup in Fig. 1 contains Bence Jones antiserum which also has antibodies to gamma globulin and albumin. Cup 2 contains Bence Jones proteose, cup 3 eluted antibody from the red cells of patient No. 2 (Table 1)

TABLE 1.

Patients	Direct Coombs test	Haemolysis	Eluent from Red Cells		Remarks
			No. of bands	Type of protein present	
*1	+	Active	2	Beta globulin	Cold agglutinin
2	+	Active	2	Gamma globulin + Albumin	Cold agglutinin
3	+	Active	1	Gamma globulin	Cold agglutinin + cryoglobulin
4	Weakly +	Active	1	?Abnormal protein	
5	+	Absent	1	Gamma globulin	"Cured" acquired haemolytic anaemia
6	+	Absent	1	Gamma globulin	
7	+	Active	1	Gamma globulin	Rh incompatibility
8	+	Active	1	Gamma globulin	

* Firkin and Blackburn (1956)

and cup 4 contains isolated gamma globulin. The reaction of identity which demonstrates that the antigens are immunologically similar is shown by the continuous line formed between cups 1 and 4 and cups 1 and 3, and therefore cups 3 and 4 must contain a quantity of the same antigen, gamma globulin. The reaction of partial identity or "spur formation" shows that the antigens are closely related but are not immunologically alike. This is shown in Fig. 1 by the junction of the line between cups 1 and 2 and the line closer to cup 3 between 1 and 3. The lines join one another but do not cross and result in the formation of a "spur". In this instance, the antigens which are closely related but not alike are Bence Jones proteose and gamma globulin. The third type of reaction is that of non-identity and indicates that there is no immunological relationship between the antigens forming the lines of precipitate. This is demonstrated by the line between cups 1 and 2 and the line closer to the central cup between 1 and 3. These two lines pass straight through one another and this illustrates the reaction of non-identity between albumin and Bence Jones proteose (Oudin, 1952).

Fig. 2 illustrates the type of reaction obtained with beta and gamma globulins when anti-IV_7 serum was used and shows how beta and gamma globulins

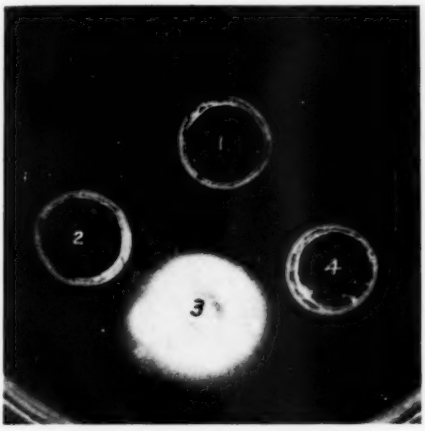

Fig. 1

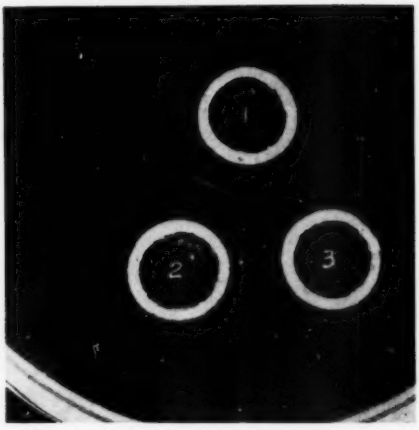

Fig. 2

Fig. 1. Cup 1 contains anti-Bence Jones rabbit serum, Cup 2 Bence Jones urine (diluted 1:7 with 0·85 p.c. saline), Cup 3 cold agglutinin (Case No. 2), and Cup 4 gamma globulin. One line is present between 1 and 2 and 1 and 4 and two lines between 1 and 3. The closer of these latter lines to Cup 3 shows a reaction of identity with gamma globulin and a "spur reaction" with Bence Jones protein. The one nearer to Cup 1 crosses both these lines, it is therefore unrelated immunologically (Oudin, 1952), and is due to albumin. The white opacity in cup 3 is due to the presence of haemoglobin in the eluent.

Fig. 2. Cup 1 contains anti-IV_7 rabbit serum, Cup 2 beta globulin and Cup 3 gamma globulin. Two lines have formed between Cups 1 and 2 and one between 1 and 3. These lines show no reaction of identity (Figs. 1 and 3) and therefore distinguish beta and gamma globulin immunologically.

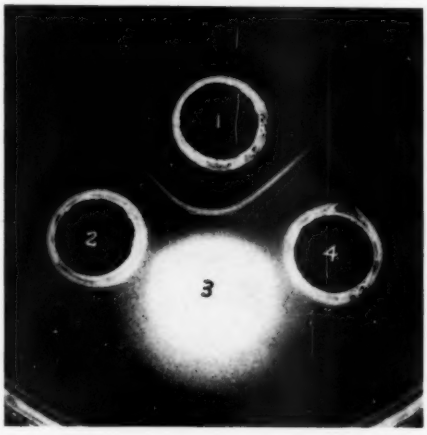

Fig. 3

Fig. 3. Cup 1 contains anti-III rabbit serum, Cup 2 gamma globulin, Cup 3 cold agglutinin from Case No. 2, and Cup 4 gamma globulin. A single line is present between Cups 1 and 2 and 1 and 4, but two lines have formed between 1 and 3. The closer of the latter to Cup 1 has joined the lines between 1 and 2 and 1 and 4 to form one continuous line. This showed that the eluted cold agglutinin possesses an antigen which is immunologically identical with gamma globulin (Oudin, 1952). The other line between 1 and 3 is closer to Cup 3 and is due to albumin. The white opacity in Cup 3 is due to the presence of haemoglobin in the eluent.

can be distinguished by this method. Cup 1 contains IV_7 antiserum, cup 2 beta globulin and cup 3 gamma globulin. Two lines of precipitate have formed with beta globulin and one with gamma globulin.

Fig. 3 shows how protein eluted off the red cell can be identified by this method. Cup 1 contains anti-III serum, cups 2 and 4 gamma globulin, cup 3 eluted protein from patient No. 2's red cells (Table 1). Lines have formed between cup 1 and the peripheral cups and have joined together to form a single continuous line. This is another example of a reaction of identity and means that the eluted protein contains an antigen immunologically similar to normal gamma globulin.

Detailed results are recorded in Table 1 and it is seen that in one instance two proteins were adsorbed on to the red cells. The two proteins in this case were immunologically and electrophoretically identical with normal beta globulin (Firkin and Blackburn, 1956). In another instance Case No. 2 two protein lines were also found, one of which was identical with gamma globulin (Fig. 1) and the other with albumin. In this case it is most probable that the albumin represented a contaminant due to insufficient washing of the cells. Evidence for the presence of a single adsorbed protein was found in the remainder of the samples. In Case No. 4, the line formed by the eluent was broad and diffuse and so was quite unlike the sharp lines formed in other instances. It is possible that this represented a false positive reaction rather than the presence of an abnormal protein although similar lines have not been encountered in eluates from normal red cells so far examined and the presence of a weakly positive Coombs test confirmed the presence of an adsorbed protein.

DISCUSSION.

These findings emphasise the non-specificity of the Coombs reaction and show that a positive Coombs test means only that a protein capable of cross-reacting with anti-human serum has been adsorbed on to the red cell. Coombs serum as supplied by the Commonwealth Serum Laboratories contains antibodies to beta and gamma globulins (Firkin and Blackburn, 1956), although the titre of the former appears to vary. The differing types of protein adsorbed on to the red cells in the small number of patients so far examined makes one wonder whether the Coombs-negative acquired haemolytic anaemias might be associated with an adsorbed protein which does not happen to cross-react with the Coombs serum being used.

The number of bands obtained with Ouchterlony's method on the red cell eluate may not represent the total number of antigens present, i.e., one band may represent more than one antigen. It can only be deduced from the findings that at least one or at least two proteins were adsorbed to the red cells in the appropriate cases.

The significance of the presence of two proteins attached to the red cells is difficult to interpret and may be related to the phenomenon of cold agglutination or may be due to the presence of complement (Dacie, Crookston and Christenson, 1957) as well as the red cell antibody. In Case No. 4 the adsorbed protein only reacted weakly with our antiserum and so it might be an abnormal protein. These findings may explain the association of a weakly positive Coombs test with a haemolytic anaemia since the adsorbed protein might be a beta globulin or an abnormal one rather than a "normal" gamma globulin. Furthermore, the frequent lack of correlation between the Coombs test and the response to treatment may be due to the removal of only one of the adsorbed proteins, the other remaining attached to the red cell and causing a strongly positive Coombs test although haemolysis has ceased or been reduced. Unfortunately a suitable patient has not been available to test the latter hypothesis.

SUMMARY.

Eight patients with Coombs-positive acquired haemolytic anaemia of varying aetiology have been studied. It has been shown that the protein attached to the red blood cell and presumably causing the positive Coombs reaction was a gamma globulin in most instances. In one case, however, the attached protein was probably an abnormal one and in another it was a beta globulin. The significance of these findings has been discussed.

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A COMPARISON OF THE PHARMACOLOGICAL PROPERTIES OF THE THREE ISOMERIC AMINOPYRIDINES

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Our interest in the aminopyridines derives from studies of the distribution of pressor activity amongst substances which are related chemically to S-methyl *iso*thiourea, $CH_3SC(:NH)NH_2$. It has been shown (Fastier and Smirk, 1947; Fastier, 1948; Fastier and Reid, 1952) that various other compounds of formula $XC(:NH)NH_2$ resemble S-methyl *iso*thiourea closely in their pharmacological properties, the chemical structure of the substituent *X* attached to the amidine group not appearing to be of great importance so long as it permits the compound to ionize freely and give a cation of small size. Nevertheless, short-chain aliphatic amines show little resemblance to short-chain amidine derivatives in their pharmacological properties though they are of comparable basicity ($pK_a \approx 10$).

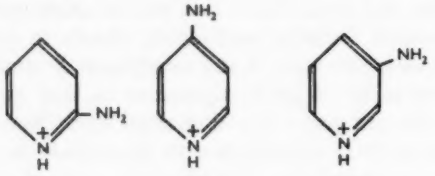
When this problem was discussed previously (Fastier and Reid, 1952), two possible explanations were advanced: (1) the amidinium group might have a specific affinity for certain tissue receptors, and (2) the much greater pharmacological activity of short-chain amidine derivatives might be due merely to their being adsorbed more strongly than the corresponding amines.

The second explanation is supported by the fact that aliphatic amines with 5-7 carbon atoms in their side-chains resemble short-chain amidine derivatives far more closely in their pharmacological properties than do amines with short side-chains — it could be argued that the extra CH_2 groups in the side-chain of the higher homologues compensate for the smaller affinity of the substituted ammonium group (as compared with the amidinium group) for receptors.

We have sought more definite evidence by comparing the properties of the isomeric aminopyridines. The 2-substituted derivative (2-AP, I) can be regarded as a cyclic amidine derivative, and it has already been shown to resemble S-methyl *iso*thiourea fairly closely in its circulatory properties (Fastier, 1948; Fastier and Reid, 1948). The 4-substituted derivative (4-AP, II), on the other hand, is not an amidine derivative. However, according to the well-known principle of vinylogy, 4-AP should resemble 2-AP closely in most other respects; and this is known to be the case (Elderfield, 1950). Both 2-AP and 4-AP are fairly strong bases, in contrast to 3-aminopyridine (3-AP, III). Whereas the

properties of the NH_2 group of 3-AP are those of an aromatic amine and the base easily forms a dihydrochloride, the NH_2 group of both 2-AP and 4-AP behaves like that of an amidine group and these bases form only a monohydrochloride under ordinary conditions. The molecules of 2-AP and 4-AP are obviously resonance hybrids. They should not differ much in adsorptability.

We hoped that a comparison of various pharmacological properties of 2-AP and 4-AP would reveal either a close resemblance or little resemblance between the compounds. Finding that there was little resemblance could be taken to indicate that the spatial arrangement of the two nitrogen atoms of 2-AP was of fundamental importance for the possession of pharmacological activity like that displayed by S-methyl *iso*thiourea, whereas finding a close resemblance could be taken to indicate that possession of an amidinium group is of importance merely because of its effect on the properties of the molecule as a whole, e.g., basicity, adsorptability.



(I) (II) (III)

METHODS.

Cats were anaesthetized with chloralose (usually 70 mg./kg.). Blood pressure was recorded from a carotid artery. The method employed for recording respiratory movements was similar to that described by Gaddum (1941). Drug solutions were injected into a saphenous vein.

Effects on blood vessels were studied by perfusing rat hindquarters at a constant rate (Fastier and Smirk, 1947). Both Ringer-Locke and Ringer-Tyrode solution were used for experiments on isolated organs. The Ringer solution was aerated with oxygen containing 5 p.c. carbon dioxide.

Strips of rat ileum were mounted in a small organ bath, the solutions in which were changed automatically as in the apparatus described by Gaddum and Lembeck (1949). It was arranged in most experiments that the ileal strip was in contact with Ringer containing carbachol for 35 sec. and with plain Ringer for 65 sec., both phases of the cycle commencing with a 13 sec. wash period. The effects of the aminopyridines on carbachol-induced contractions were studied by injecting them into the organ bath immediately after the wash with plain Ringer. Their effect on tone alone was observed by omitting the carbachol wash and changing the contents of the bath at a slow steady rate.

Rat diaphragm preparations were set up as described by Bülbring (1946) except that the bath temperature was kept at 25° C. The muscle was stimulated either directly or through the phrenic nerve. 'Square wave' electrical shocks of 1 msec. duration and of sufficient intensity to produce a maximal contraction were applied through platinum electrodes at a frequency of 6 per min.

Female white mice were used for toxicity tests. The drugs were normally given intraperitoneally. When they were given by the intracerebral route, the method recommended by Haley and McCormick (1957) was followed. The syringe was mounted vertically in the clamp of a micrometer screw gauge, which was held in turn to a heavy clamp by a rack and pinion. The syringe plunger was firmly attached to the end of the micrometer by elastic bands. Small amounts (0·01-0·05 ml.) of drug solutions were injected by rotating the micrometer spindle. To prevent the needle being inserted too deeply, a stop was placed on its shaft 1 cm. from the tip.

All the pyridine derivatives tested were purchased from L. Light & Co. The bases were brought into solution with hydrochloric or acetic acid.

RESULTS.

Effects on anaesthetized cats. All three aminopyridines raised arterial blood pressure when injected intravenously in a dose of 1 mg./kg. The effects of 2-AP and of 4-AP were prolonged; it was not unusual for the blood pressure to be still more than 20 mm. or more above its original level 30 min. after the injection of the drug. In experiments in which all three compounds were tested on the one cat, 3-AP produced considerably smaller rises of blood pressure than did 2-AP and 4-AP (Table 1). The effect of these compounds on heart rate was variable. They often caused a striking increase in respiratory movement, usually accompanied by other signs of central stimulation such as twitching of the head and limbs.

TABLE 1.

Pressor responses of anaesthetized cats to the intravenous injection of aminopyridines (1 mg./kg.).

	2-AP	3-AP	4-AP
Cat 1	20, 20 mm.	12, 22 mm.	70 mm. Hg.
Cat 2	30	18, 32	32
Cat 3	38	8, 10	40
Cat 4	40	0	60
Average	30 mm. Hg.	15 mm. Hg.	50 mm. Hg.

Effects on perfused rat blood vessels. In the main set of experiments hindquarters were perfused with Ringer-Locke solution at 35-37° C. and the aminopyridines were injected in 1 mg. doses. Compounds were given no more frequently than once every 10 min. Under these conditions the response to successive doses of a compound did not diminish ('tachyphylaxis'). When Ringer-Tyrode was used in place of Ringer-Locke solution, the vasoconstrictor effect of the aminopyridines (but not of adrenaline) rapidly diminished in the course of an experiment. Pithing the hindquarters had no obvious effect on the response to the aminopyridines.

The average increase in perfusion pressure (indicating vasoconstriction) obtained with 2-AP was 15·7 mm. of Hg, whereas that obtained with 3-AP

under comparable conditions was 6·5 mm. and that obtained with 4-AP was 16·6 mm. Since the standard errors of these means were 1·75, 0·84 and 2·48 respectively, the vasoconstrictor effects of 2-AP and 4-AP were significantly ($P < 0·01$) greater than those of 3-AP.

In 5 experiments the aminopyridines were given within 10 min. of the injection of small doses of adrenaline but after the vasoconstrictor effect of the latter had worn off. The average vasoconstrictor response to 3-AP was approximately doubled when this was done but the response to 2-AP or 4-AP was not enhanced to any noteworthy extent. Curiously enough, the vasoconstrictor responses to all three aminopyridines were enhanced when they were given after amounts of ergotoxine sufficient to cause a prolonged desensitization to adrenaline. The mean rises of pressure obtained under these conditions with 2-AP, 3-AP and 4-AP respectively were 38·5, 28·3 and 30 mm. (compared with 15·7, 6·5 and 16·6 mm. in untreated preparations).

Effects on isolated rat ileum. Ileal strips which had gradually relaxed in plain Ringer were strongly contracted when 2-AP was added to the inflowing Ringer to give concentrations of the order of 0·005 M. Much smaller increases in tone were produced by 3-AP and 4-AP in the same concentration and the effect of 3-AP was transient. With concentrations of 0·001 M., effects on tone were negligible.

In the remaining experiments the aminopyridines were administered while the strips were being stimulated periodically by washing them with Ringer containing carbachol (1-5 μg./ml.). The aminopyridines were added in known concentration to both the plain and the carbachol-containing Ringer for 6-12 cycles, after which the ordinary solutions were used again. Both 2-AP and 4-AP in 0·005 M. concentration decreased the amplitude of the carbachol contractions by 50 p.c. or more, though they themselves sometimes increased tone at the same time. These effects were easily reversed by changing back to plain Ringer. Much more variable responses were obtained when 3-AP was given in concentrations of from 0·002-0·01 M. Sometimes it increased tone without affecting the amplitude of the carbachol contractions, sometimes it increased the carbachol contractions without affecting tone, sometimes it increased or decreased both.

Effects on rat diaphragm. Both 2-AP and 4-AP produced a gradual enhancement of the contraction produced by a slightly supermaximal shock when added to the muscle bath in a concentration of 0·005 M. Over a period of 4 min. they increased the magnitude of the response by 50-250 p.c. This effect was not readily reversible. The preparation had to be washed repeatedly over a period of 10-20 min. to get the amplitude of the contractions back to normal. However, reproducible results could be obtained with the one compound. The enhanced response was obtained with 2-AP and 4-AP irrespective of whether the shocks were applied to the phrenic nerve or directly to the muscle. When

tested in the same concentration 3-AP had a much more variable effect; in some experiments it potentiated the effect of supermaximal electrical shocks almost as strongly as 2-AP, but usually it had comparatively little effect.

Effects on unanaesthetized mice. All three aminopyridines in lethal doses produced increased excitability to sound and touch, tremor, then clonic convulsions, tetany and death. The convulsions were almost always fatal. Since treated mice excited each other when kept in the same cage, mice were put in separate beakers for LD_{50} determinations. The mortality data for batches of 10 mice treated with different doses of the aminopyridines are given in Table 2. It was calculated from these figures that the LD_{50} for 2-AP was 35

TABLE 2.

Mortality data for mice treated with aminopyridines.

The compounds were given intraperitoneally, the doses are in mg./kg., and the number of deaths are those for a batch of 10 mice treated with that particular dose of the compound.

2-AP		3-AP		4-AP	
Dose	Deaths	Dose	Deaths	Dose	Deaths
25	1	20	1	8	4
30	2	24	—	9	5
35	5	28	5	10	9
40	8	32	7	—	—

mg./kg., for 3-AP 28 mg./kg., and for 4-AP 9 mg./kg. (for intraperitoneal injection). The effects produced by the isomers before the terminal strychnine-like convulsions differed appreciably: mice treated with 2-AP ran about madly, those treated with 3-AP developed tremors and a tendency to walk backwards, while those treated with 4-AP were at first affected most in the limbs with the result that they progressed in short hops.

When control injections of saline were given by the intracerebral route the mice were dazed for about a minute, then they resumed normal activity except for a few which were probably injured by faulty placing of the needle and which exhibited such symptoms as giddiness with unilateral stretching or splaying of the limbs, progressing in circles, or rolling over continuously with inability to stand upright. The effects produced by intracerebral injections of the aminopyridines were essentially similar to those produced by intraperitoneal injections of the same compounds. All three isomers produced clonic convulsions and death in less than 30 sec. when given in a dose of 100 μg. Both 2-AP and 4-AP produced characteristic effects in a dose of 50 μg. but the effects of 3-AP were minimal at this dose level. The corresponding minimally effective doses for 2-AP and 4-AP were 20 μg. and 10 μg. respectively.

All three isomers possess appreciable analgesic activity. This aspect of the pharmacology of aminopyridines will be dealt with in another paper.

DISCUSSION.

Evidence concerning the site of the pressor action of S-methyl *iso*thiourea and other short-chain amidine derivatives has been presented in earlier papers (Fastier and Smirk, 1947; Fastier, 1948, 1949; Fastier and Reid, 1952). It seems probable that these compounds raise blood pressure mainly by virtue of a direct stimulant action on the smooth muscle of blood vessels. The vasoconstrictor action of S-methyl *iso*thiourea is not typically 'sympathomimetic' in that it is not antagonized appreciably by such anti-adrenaline drugs as ergotoxine, piperoxane and tolazoline. The effects of S-methyl *iso*thiourea on various nerve-muscle preparations resemble those of the potassium ion or the 'nicotinic' effects of acetylcholine rather than those of adrenaline.

The experiments just performed indicate that similar activity is possessed, not only by 2-AP, which shows an obvious structural resemblance to S-methyl *iso*thiourea and other short-chain amidine derivatives, but also by 4-AP and (though to a less extent) by 3-AP, neither of which contains the —N:C(NH_2)— group. We therefore conclude that the spatial relationship of the two nitrogen atoms of the aminopyridines group is not of fundamental importance for conferring pressor activity and allied pharmacological properties.

A number of other investigators have described pharmacological effects of 2-AP, but the properties of 3-AP and 4-AP have received scant attention. Dingemanse and Wibaut (1928) observed that 3-AP resembles 2-AP in producing strychnine-like convulsions in frogs. Shaw and Bentley (1955) tested 2-AP and 4-AP, along with various other organic bases, on rabbits treated with morphine and found both to be respiratory stimulants

The properties of all three aminopyridines have been studied recently by von Haxthausen (1955). Most of his findings resemble ours closely. Thus his values for the LD_{50} of 2-AP, 3-AP and 4-AP given subcutaneously to mice are 70, 30 and 5 mg./kg. respectively, while ours for the same compounds given by intraperitoneal injection are 35, 28 and 9 mg./kg. respectively. He, too, found the convulsant dose to lie close to the lethal dose and the toxic effects of the isomers to be very similar, death being preceded by intense excitement and convulsions. Like von Haxthausen, we have found 4-AP to produce larger rises of blood pressure in anaesthetized cats than 2-AP when given in the same dosage. Our results differ from his, however, in that we have found 3-AP to produce smaller rises of blood pressure than 2-AP. We have found likewise that the vasoconstrictor action of 3-AP on perfused rat blood vessels is distinctly weaker than that of 2-AP and 4-AP, that 3-AP is by far the weakest of the isomers in potentiating the effect of maximal electrical stimuli on the rat diaphragm preparation, and that the effects of 2-AP on isolated rat ileum are reproduced more closely by 4-AP than by 3-AP.

The aminopyridines differ from short-chain amidine derivatives like S-methyl *iso*thiourea in having a much more intense action on the central nervous system. Perhaps this is due to more rapid penetration of the blood-brain barrier

on the part of the aminopyridines. It seems significant that the minimum doses needed for the production of convulsions when the aminopyridines were given by the intracerebral route (1, 2·5 and 0·5 mg./kg. for 2-AP, 3-AP and 4-AP respectively) were not very much smaller than the corresponding doses for the intraperitoneal route (15, 10 and 3 mg./kg.).

SUMMARY.

The pharmacological resemblance between 2-aminopyridine and 4-aminopyridine is very close. Both isomers produce sustained rises of blood pressure in anaesthetized cats and stimulate respiratory movement when they are given intravenously in a dose of 1 mg./kg. Both constrict perfused rat blood vessels even when enough ergotoxine is present to suppress almost completely the vasoconstrictor action of adrenaline. Both increase the tone of isolated strips of rat ileum, both potentiate the maximal contractions of the rat diaphragm preparation elicited by electrical shocks applied either to the phrenic nerve or directly to the muscle, and both produce strychnine-like convulsions in unanaesthetized mice when they are injected in near-lethal doses.

The remaining isomer, 3-aminopyridine, resembles the other two in most of the above respects, but usually acts less strongly.

Because 2-aminopyridine, which is an amidine derivative, shares pressor activity and other characteristic properties with its isomers, which do not possess a —N:C(NH_2)— group, it is concluded that the pharmacological resemblance between 2-aminopyridine and such short-chain amidine derivatives as S-methyl *iso*thiourea is not due essentially to possession of an amidine group by all these compounds.

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BLOOD GROUP ANTIGENS IN WHITE CELLS

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Although the blood group antigens were originally found in red cells, a number of authors have demonstrated the presence of some of these antigens in tissue cells and platelets (Ashhurst, Bedford and Coombs, 1956). It was natural to seek these antigens in the circulating white cells, but here a difficulty was encountered in that leucocytes tend to undergo spontaneous agglutination. This tendency no doubt was the reason for some non-specific agglutination reported by Whyte and Yee (1956) and by Moeschlin and Schmidt (1954).

A method which avoids spontaneous agglutination of the leucocytes was used by Berroche, Maupin, Hervier and Dausset (1955). In this method the blood group antibody was absorbed specifically by the leucocytes, the antibody was eluted at 56° C. and demonstrated by the specific agglutination of red cells.

It is the purpose of this paper to describe a modification of the absorption-elution method in which elution of the antibody from the leucocytes takes place at room temperature. This method is somewhat simpler and yields specific results.

In addition, a method for preparing white cells and for separating white cells into polymorphs, lymphocytes and monocytes will be described.

MATERIALS AND METHODS.

White cell suspension.

The white cells were obtained from a 500 ml. bottle of sedimented or centrifuged blood. Blood less than 24 hours old was used if the different varieties of white cells were to be separated; otherwise blood less than four days from collection was satisfactory. The supernatant plasma was aspirated into another bottle as in the preparation of concentrated cells (Walsh, 1953). The aspirating needle was then inserted further into the bottle and the buffy coat layer together with a small amount of residual plasma and some red blood cells was aspirated into a separate bottle. The contents of this bottle were poured into centrifuge tubes which were centrifuged at 1500 r.p.m. for 10 minutes. The buffy coat layers from these tubes were then pooled and after mixing were centrifuged at 1500 r.p.m. for 10 minutes. The leucocyte layer was carefully removed and pipetted into a centrifuge tube containing 10 ml. physiological saline.

The tube was inverted to mix the contents and the cells were allowed to sediment. The white cells formed clumps and settled to the bottom of the tube, leaving the red cells and platelets dispersed through the saline. The supernatant saline, red cells and platelets were removed with a pipette. More saline was added and after mixing the cells were allowed

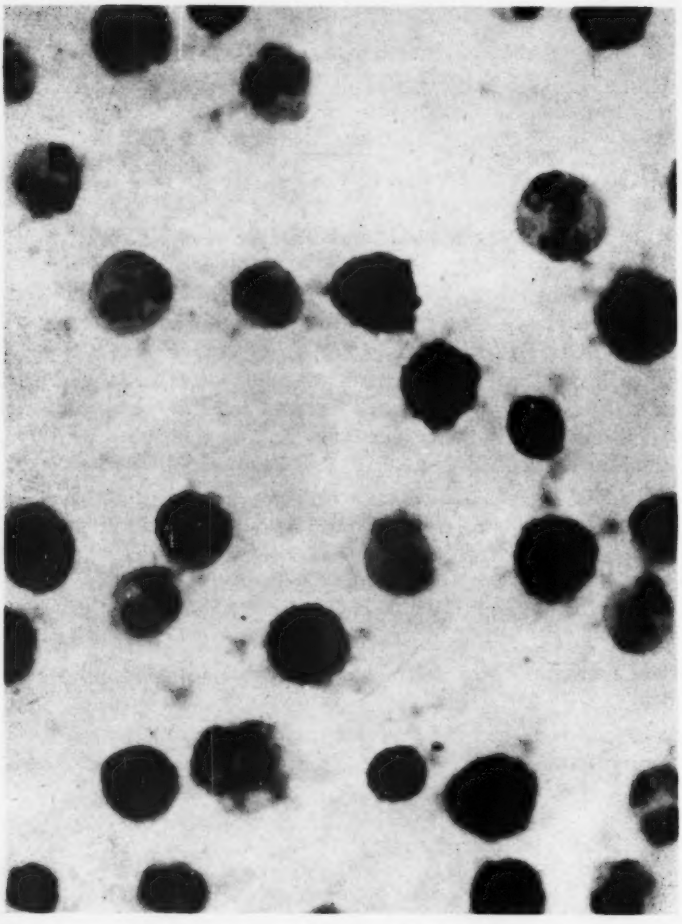

Fig. 1. Photomicrograph of a stained film of the white cell preparation.

to sediment again. When this sedimentation process had been repeated three times a white cell preparation containing less than one red cell per thousand leucocytes and no visible platelets was obtained. Fig. 1 shows a stained film of such a white cell preparation.

Separation of different varieties of white cells.

The method was based on the fact that polymorphs have a higher specific gravity than lymphocytes, which themselves have a higher specific gravity than monocytes. They can be separated by centrifugation in albumin solutions of different concentrations. These solutions were prepared from 25 p.c. salt-poor human albumin obtained from human serum by Cohn's fractionation process. The albumin was dialysed against distilled water for eight hours and then lyophilised in ampoules. Solutions containing 35 p.c. and 50 p.c. albumin in physiological saline were prepared from the dried albumin.

Standard Wintrobe haematocrit tubes were used for the separation and care was taken to ensure that the tubes were horizontal while being centrifuged by inserting them into pressure tubing in the centrifuge cups.

It was found impossible to separate the different varieties of white cells in one Wintrobe tube. Accordingly, monocytes, lymphocytes and polymorphs were obtained from aliquots of the white cell suspension in three separate tubes. The tubes were prepared as shown in Fig. 2.

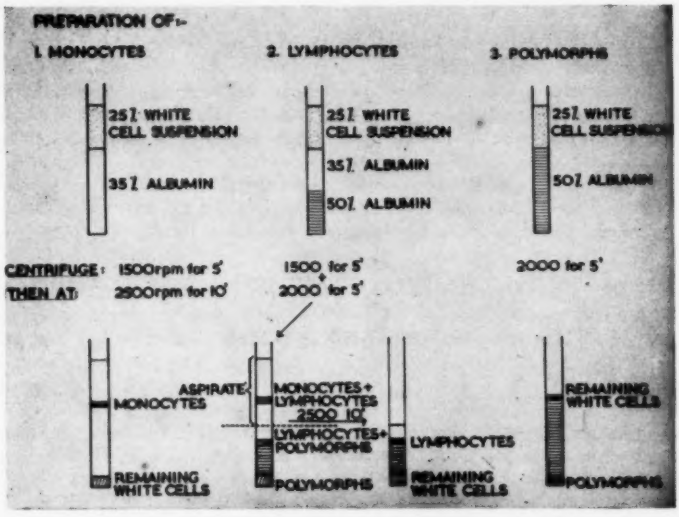

Fig. 2. Method of separating white cells shown diagramatically. The upper portion of the diagram shows the haematocrit tubes before centrifugation. The lower portion of the diagram shows the haematocrit tubes after centrifugation.

Monocytes. A 4 cm. column of 35 p.c. albumin was placed in the first tube and a 2 cm. column of a 25 p.c. white cell suspension in saline was placed on the albumin layer. The tube was centrifuged at 1500 r.p.m. (250 *g*) for five minutes and then at 2500 r.p.m. (700 *g*) for 10 minutes. The monocytes accumulated at the saline/albumin interface, while the other white cells passed through the albumin to the bottom of the tube. The monocyte layer was removed with a Pasteur pipette. A differential count performed on such a preparation gave the following results: Monocytes 89·5 p.c.; lymphocytes (possibly including some small monocytes), 9 p.c.; polymorphs 2·5 p.c.

Lymphocytes. A 2 cm. column of 50 p.c. albumin was placed at the bottom of the second haematocrit tube and a 2 cm. column of 35 p.c. albumin was placed on it. Finally, a 2 cm. column of 25 p.c. white cell suspension in saline was placed on the 35 p.c. albumin. After the tube had been centrifuged at 1500 r.p.m. for 5 minutes and then at 2000 r.p.m. (450 *g*) for a further 5 minutes, the white cells at the saline/albumin interface were removed. The tube was then spun at 2500 r.p.m. for a further ten minutes. Lymphocytes accumulated at the interface between the 35 p.c. and 50 p.c. albumin solution and were removed with a Pasteur pipette. A differential count performed on one preparation gave the

following results: Lymphocytes 84 p.c.; basophils 6·5 p.c.; polymorphs 5 p.c.; eosinophils 4·5 p.c.

Polymorphs: A 4 cm. column of 50 p.c. albumin was placed at the bottom of the third haematocrit tube and a 2 cm. column of 25 p.c. white cell suspension was placed on it. The tube was centrifuged at 2000 r.p.m. for 5 minutes. The polymorphs were thrown to the bottom of the tube and were collected with a Pasteur pipette, after removal of the supernatant cells and fluid. The results of a differential count on one such preparation were as follows: Polymorphs 90·5 p.c.; lymphocytes 6·5 p.c.; eosinophils 3 p.c.

Notes.

One difficulty sometimes encountered was the tendency of the leucocytes to slide down the side of the tube instead of accumulating at the interface or passing through the albumin. It was found that this tendency could be counteracted by preliminary centrifugation of each tube. The speed of the centrifuge was rapidly raised to 1500 r.p.m. and then stopped 30 seconds after commencement of the operation. The tube was then twisted through 180° in the centrifuge cup.

The different centrifuge speeds were determined by trial and error. It was found that if low speeds were used in the first instance the separation of the different varieties of white cells was more effective than if the higher speeds were used throughout.

Typing sera.

Anti-A and anti-B typing sera were high titre sera prepared by immunising volunteers with A and B blood group substances. The anti-D serum was obtained from an immunised woman and contained a complete antibody of high titre. Rabbit anti-M and anti-N sera were obtained from the Ortho Pharmaceutical Corporation. The anti-S serum was a human serum with a titre of 1/16 in saline. The anti-Tj(a) serum was obtained from a Tj(a−) blood donor and her white cells were used as negative controls. Two anti-P sera were used, one occurring naturally in a P negative donor, the other obtained by absorption of anti-Tj(a) with P negative cells (Sanger, 1955). Both were active on red cells at room temperature. The Lewis antibody used was active at room temperature to a titre of 1/4.

Red cells.

All red cells were washed three times with saline and a 3 p.c. suspension in saline prepared.

ABO grouping.

One drop of the white cell preparation was pipetted into each of two centrifuge tubes. Five drops of anti-A serum were added to one of the centrifuge tubes and five drops of anti-B serum to the other. The white cells were left in contact with the serum for one hour at room temperature, the tubes being occasionally shaken to disperse the cells. The white cells were then washed three times with physiological saline. The button of cells obtained after the final washing was dispersed in 4 or 5 drops of saline and divided into two equal portions, which were placed in ⅝ in. × 3 in. test tubes. One drop of 3 p.c. thrice-washed group A red cells was added to one tube and one drop of 3 p.c. thrice-washed group B red cells to the other. The tubes were shaken gently and then placed in a water bath at 37° C. for 30 minutes. A portion of the contents of each tube was removed with a pipette, placed on a microscope slide and examined for agglutination of the red cells.

In all preparations the white cells showed a tendency to clump non-specifically and some red cells were often incorporated in the clumps. These mixed red and white cell clumps were ignored and the red cells lying free in the preparation were examined for the presence or absence of agglutination.

Eluates were prepared at different temperatures from the washed white cell preparations. The cell-free eluates were tested against A and B red cell suspensions.

Rh grouping.

One drop of the white cell preparation was added to 10 drops of anti-D serum in a centrifuge tube. In different experiments the mixture was placed at 37° C., at bench temperature, and at 6° C. for one hour. The cells were then washed twice with large volumes of saline. The washed white cells were divided into two equal parts which were placed in ⅝ in. × 3 in. test tubes. Rh positive (D positive) red cells were added to one tube and Rh negative (D negative) red cells to the other. The tubes were placed at 37° C. for 30 minutes and then centrifuged for one minute at approximately 1000 r.p.m. The button of cells was examined microscopically for red cell agglutination. In some experiments eluates were prepared by heating the washed white cells to 56° C. for 5 minutes. These eluates were tested against Rh positive and Rh negative red cells.

MNS, P, Le(a), Tj(a) grouping.

The technique was similar to that used in ABO grouping except that the white cells were washed twice and not three times. Cold saline (6°-10° C.) was used when washing white cells which had been in contact with anti-Le(a) or anti-P sera. Eluates were prepared from the washed white cells by heating at 56° C. for 5 minutes.

RESULTS.

ABO grouping. Red cell agglutination was present as shown in Table 1. White cells from group A subjects absorbed anti-A but not anti-B, so that the addition of red cells to the washed preparation produced agglutination of A red cells but not of B red cells (Figs. 3 and 4). Similarly, white cells from group B subjects absorbed anti-B while group AB white cells absorbed both anti-A and anti-B. Group O white cells did not absorb either anti-A or anti-B. The separate polymorph, lymphocyte and monocyte preparations gave similar re-

TABLE 1.
ABO grouping.

Blood group of white cell donor	White cells exposed to	Red cells added	Red cell agglutination
A	anti A	A	+++
	anti A	B	–
	anti B	A	–
	anti B	B	–
B	anti A	A	–
	anti A	B	–
	anti B	A	–
	anti B	B	+++
AB	anti A	A	+++
	anti A	B	–
	anti B	A	–
	anti B	B	+++
O	anti A	A	–
	anti A	B	–
	anti B	A	–
	anti B	B	–

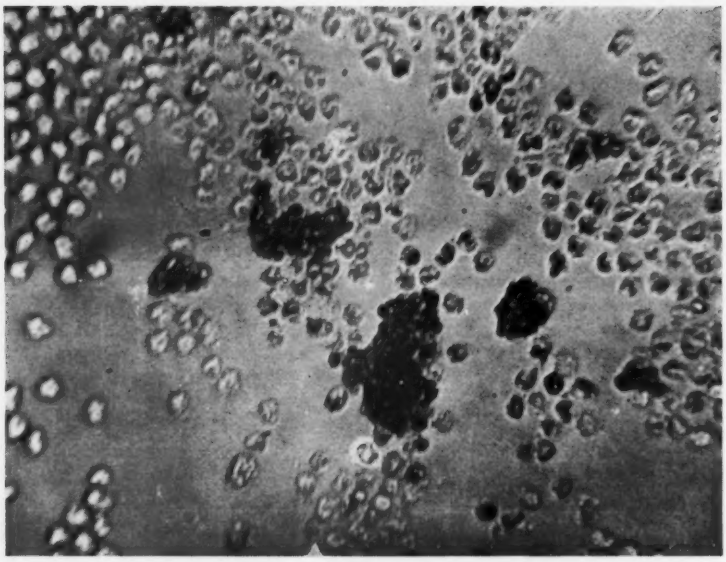

Fig. 3. Positive result: clumping of group A red cells in the presence of eluted antibody and white cells from a group A donor.

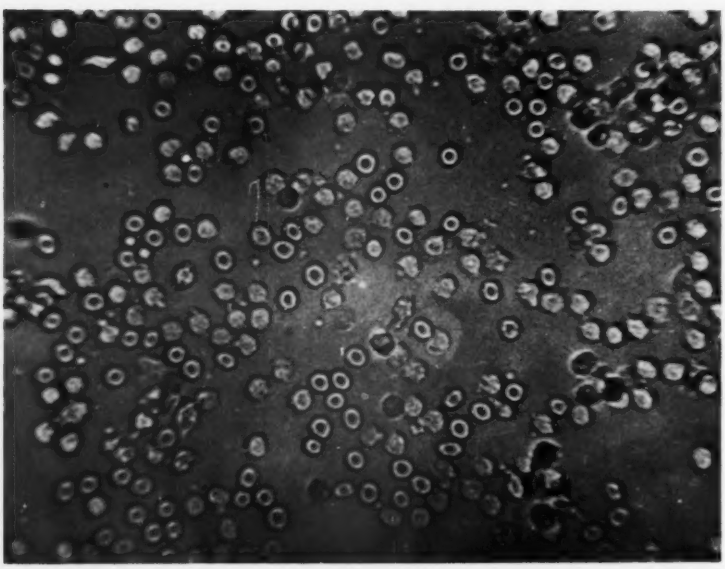

Fig. 4. Negative result.

sults. When the eluates obtained from white cells were added to red cell suspensions, specific red cell agglutination occurred.

The effect of temperature on the elution of anti-A from A white cells is shown in Table 2. It will be seen that at bench temperature (20° C.) antibody is eluted and that the amount of antibody eluted increases with increasing temperature.

TABLE 2.

Effect of temperature on elution.

Titre of eluate	Temperature of elution			
	10° C.	20° C.	37° C.	56° C.
1/1	+++	+++	+++	+++
1/2	+++	+++	+++	+++
1/4	++	+++	+++	+++
1/8	–	+++	+++	+++
1/16	–	++	+++	+++
1/32	–	+	+++	+++
1/64	–	–	++	++
1/128	–	–	w	+
1/256	–	–	–	w
1/512	–	–	–	

+++, ++, etc., indicate the degree of red cell agglutination.

Rh grouping. No anti-D appeared to be absorbed when white cells were mixed with antibody at 37° C. On the other hand, white cells from Rh positive and Rh negative individuals absorbed antibody non-specifically at 6° C. This was shown by agglutination of added Rh positive red cells but not of added Rh negative red cells, irrespective of whether the white cells were derived from Rh positive or Rh negative subjects.

Tj(a) grouping. White cells from five Tj(a) positive subjects and one Tj(a) negative subject were tested with anti-Tj(a) serum. The results in Table 3 show that specific absorption and elution of anti-Tj(a) took place.

TABLE 3.

Tj(a) grouping.

Blood group of white cell donor	White cells exposed to	Tj(a) positive red cells added	Tj(a) negative red cells added
Tj(a) positive	anti-Tj(a)	+++	–
Tj(a) positive	anti-Tj(a)	+++	–
Tj(a) positive	anti-Tj(a)	+++	–
Tj(a) positive	anti-Tj(a)	+++	–
Tj(a) positive	anti-Tj(a)	+++	–
Tj(a) negative	anti-Tj(a)	–	–

S grouping. The results of S grouping are shown in Table 4. It appears that S positive white cells absorbed and eluted anti-S specifically.

TABLE 4.
S grouping.

Blood group of white cell donor	White cells exposed to	Red cells added	Red cell agglutination
S positive	anti-S	S positive	+++
	anti-S	S negative	–
S negative	anti-S	S positive	–
	anti-S	S negative	–

M, N, P and Le(a) grouping. Specific absorption of anti-M, anti-N, anti-P and anti-Le(a) antibodies could not be demonstrated.

DISCUSSION.

The results reported in this paper show that the A, B, S and Tj(a) antigens are present in the white cells. The A and B antigens were demonstrated in the polymorph, lymphocyte and monocyte preparations. The method used for their demonstration depends on the absorption and spontaneous elution of the antibodies from white cells. It is concluded that the union of the antibody with the antigen on the white cells is not firm. Specific agglutination of red cells added to the eluate demonstrates the presence of the antibody in the eluate. This technique does not depend on the direct agglutination of white cells which often occurs non-specifically to give unreliable results.

The presence of the blood group antigens in white cells was thought probable because these antigens have been found in a number of different somatic cells. The A and B antigens have been found in cutaneous epithelial cells (Coombs *et al.*, 1956), platelets (Ashhurst *et al.*, 1956) and in alcoholic extracts of tissue cells (Boorman and Dodd, 1943). The Rh antigens have been found in liver, kidney, spleen, heart and brain of infants who have died from haemolytic disease of the newborn (Brading and Walsh, 1954). The Tj(a) antigen has been found in platelets (Ashhurst *et al.*, 1956). Unfortunately the technique used for this work is not suitable for the demonstration of leucagglutinins which various workers have shown to be formed against white cells by immunisation. Failure to show the presence of Rh (D), M, N, Lewis or P antigens raises the question, at present unanswerable, of whether these antigens are present in the leucocytes or whether the technique is unsuitable for demonstrating their presence.

SUMMARY.

A method is described for preparing white cells almost free of red cells and platelets, and for separating these white cells into polymorph, lymphocyte and monocyte preparations.

A, B, S and Tj(a) antigens were demonstrated in white cells by a technique in which white cells from an individual of a certain blood group absorb the corresponding antibody and subsequently release it into saline. This eluate agglutinated only red cells belonging to the same blood group as the white cells.

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THE MEASUREMENT OF CREATININE IN PLASMA AND URINE

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The measurement of creatinine should be of considerable importance in clinical practice and in research investigations. Creatinine is produced in almost constant daily amounts proportional to the muscular mass of the body, and is constantly and readily excreted by the kidneys by glomerular filtration. Thus, the concentration in plasma alone is an index of renal efficiency (Efforsøe, 1957) and the renal clearance of creatinine can be used as a measure of glomerular filtration rate (Haugen and Blegen, 1953; Owen, Robson, Scandrett and Stewart, 1955). Measurement of the amount of creatinine in urine has been suggested as a means of predicting the fat-free mass of the body (Miller and Blyth, 1952) and as providing a useful reference parameter, in preference to height, weight or surface area, for cardiac output, oxygen consumption and other physiological measurements. However, extensive use of creatinine has not been made and even when used, the results have been subjected to criticism because of the common accusation that creatinine cannot be measured accurately (Milne, 1957).

Most of the methods in use for measuring creatinine rely on the Jaffé reaction which involves the development of a yellow colour with alkaline picrate (Jaffé, 1886). This is extremely reliable, but interference occurs from various non-creatinine chromogens such as protein and glucose (Haugen, 1953), and many of the methods which claim to measure creatinine really measure the total chromogen content.

By interpreting these results as "true" creatinine an error of + 5 to + 100 p.c. may be incurred (Lauson, 1951). This has been one of the major points of confusion, but can be overcome by the use of Fuller's Earth to separate by adsorption creatinine from other chromogens (Borsook, 1935; Hare, 1950; Owen, Iggo, Scandrett, and Stewart, 1954). In addition, other factors which are liable to introduce errors unless carefully controlled include temperature, pH, and protein precipitation (Lauson; Haugen and Blegen; Mandel and Jones, 1953; Owen, *et al.*, 1954). Inattention to these details may have caused faulty conclusions in the past (Smith, 1951, 1956).

We have reinvestigated the problem and present here reasonably accurate and practical methods for measuring creatinine in plasma and urine.

Materials and Methods.

Materials.

1. Sulphuric acid, 0·67N – 10 ml. of concentrated acid diluted to 500 ml. with water.
2. Sodium tungstate – A 6 p.c. solution (W/V) in water.
3. Fuller's Earth – B.D.H. (Lloyd's reagent, hydrated aluminium silicate). Each batch must be tested for satisfactory adsorption.
4. Glass measure to contain approximately 100 mg. of Fuller's Earth.
5. Oxalic acid – A saturated solution in water.
6. Sodium hydroxide, 10N – A 40 p.c. solution (W/V) in water (stock solution).
7. Sodium hydroxide, 0·75N and 0·1N (working solutions).
8. Picric acid – A saturated solution in water, kept in a dark bottle.
9. Creatinine solutions – 250 mg. of pure creatinine (B.D.H.), or 405 mg. of creatinine zinc chloride (B.D.H.) in 250 ml. of 0·1N hydrochloric acid (stock standard). Prepare a working standard each day of 0·5 ml. of stock standard plus 0·5 ml. of 0·1N sodium hydroxide diluted to 100 ml. with water.
10. Centrifuge tubes, glass-stoppered – 15 ml. and 25 ml. capacity. Plastic stoppers are also satisfactory, but cork or rubber stoppers are unsuitable with alkaline picrate solutions.
11. Spectrophotometer – except where otherwise stated, optical densities were measured in a Beckman Model DU spectrophotometer at a wavelength of 490 mμ.

Measurement of creatinine in plasma or serum.

If the plasma protein concentration is known or suspected to be lower than normal, additional steps must be taken which are described later.

Pipette 5 ml. of plasma into a large centrifuge tube, 5 ml. of distilled water and 5 ml. of 6 p.c. sodium tungstate. Mix and add 5 ml. of 0·67N sulphuric acid. Smaller quantities can be used if duplicates are not required. Shake well, remixing the contents several times in 10 minutes. Centrifuge for 10 minutes at 2,000 r.p.m. and then filter through small No. 1 Whatman papers. The filtrate should be crystal clear.

If the plasma creatinine concentration is known to be higher than 6 mg. per 100 ml., dilute the filtrate at this stage by adding 2 ml. of filtrate to 10 ml. of distilled water.

Into the duplicate glass-stoppered 15 ml. centrifuge tubes place 100 mg. of Fuller's Earth, 5 ml. of the protein-free filtrate and 0·5 ml. of saturated oxalic acid. Two standard solutions are prepared similarly. Mix several times in 5 minutes, centrifuge for 10 minutes at 2,000 to 3,000 r.p.m. and remove the creatinine-free supernatant with a fine-pointed Pasteur pipette. The test can be interrupted at this stage, if desired, for several hours.

Freshly prepare alkaline picrate colour reagent – 10 ml. of saturated picric acid and 10 ml. of 3 p.c. sodium hydroxide (0·75N), made up to 100 ml. with distilled water. To the button of Fuller's Earth in each test tube add 5 ml. of colour reagent: or less, if greater sensitivity is required. Replace the stopper and resuspend the material by shaking several times in 5 minutes. Centrifuge for 10 minutes at 2,000 to 3,000 r.p.m. Marked rises in temperature due to the centrifuge "heating-up" must be avoided. Place the tubes and a reagent blank (alkaline picrate colour reagent) in a water bath at 25° C. for 30 minutes.

Measure optical density, using the same cuvette each time, with distilled water as reference. Readings must be taken as soon as the solution is removed from the bath, before the temperature changes. Readings may be repeated at intervals to check that colour development has reached a stable plateau: at constant temperature, the colour then remains constant for many hours. If comparable readings all the year round are not required, the

solutions can be read at room temperature instead of using a constant temperature water bath: up to 60 minutes should then be allowed for colour development in cold weather.

The creatinine content of plasma is calculated by dividing the optical density of the unknown by the optical density of the standard solution and multiplying by the plasma dilution factor (4 or 24). For example:

O.D. of reagent blank = 0·201.
O.D. of unknown filtrate (1 in 4) = 0·576.
O.D. of standard solution (1 mg. p.c.) = 1·199.
O.D. of standard solution (0·5 mg. p.c.) = 0·694.

$$\text{Unknown} = \frac{0{\cdot}375 \times 4}{0{\cdot}992} = 1{\cdot}5 \text{ mg. per 100 ml.}$$

Measurement of creatinine in urine.

The above method can be used for urine, diluted 1 in 200, proceeding as with the standard solutions. However, Fuller's Earth is rarely necessary and the following simpler method is usually more convenient unless measurements are being made on plasma and a small number of urine samples concurrently.

Dilute the urine, 0·5 ml. to 100 ml. and pipette 4 ml. into a lipped test tube of 20 ml. capacity. Freshly dilute the stock creatinine solution, 0·5 ml. to 100 ml. with distilled water, and take 4 ml. of this as a standard solution. Into a third test tube pipette 4 ml. of distilled water for a reagent blank.

To each tube add 1 ml. of alkaline picrate solution prepared by adding equal parts of 3 p.c. sodium hydroxide and saturated picric acid, 10 ml. of each. Mix by inversion, using plastic or glass stoppers, and place the test tubes in the water bath at 25° C. for at least 30 minutes before measuring the optical densities as for the plasma samples. The optical density of the unknown divided by the optical density of the standard solution gives the creatinine concentration in mg./ml.

RESULTS.

During the investigations which led to the adoption of these methods for measuring creatinine in plasma and urine, it became obvious that certain features were of particular practical importance. Observations which are relevant to these features are presented here.

Fuller's Earth. Fuller's Earth is recommended on the assumption that it specifically adsorbs all the creatinine from the solution thus separating it from any other substances which might interfere with the colorimetric measurement. One reason for the necessity of this can be seen from the difference in behaviour of plasma and a simple solution of creatinine (Fig. 1). When yellow alkaline picrate is added to a solution of pure creatinine, the colour develops steadily to reach, within 30 minutes or so, a plateau where it remains constant for many hours. By contrast, when the reagent is added to protein-free plasma the colour does not become constant but continues to increase slowly for a variable period (20 to 100 minutes after the initial rapid coloration) before fading. This gradual increase is due to the presence of numerous non-creatinine chromogens such as glucose, proteins, amino-acids, ketone bodies, barbiturates and ascorbic acid (Taussky, 1956). When Fuller's Earth is used, however, the eluate behaves like a simple solution of creatinine which suggests that interfering chromogens have effectively been removed.

One indication that Fuller's Earth probably adsorbs all the creatinine from plasma and then releases it completely into the alkaline picrate solution can be seen by examination of the curves in Fig. 1. Assuming that creatinine alone would produce a plateau in the colour curve and that any progressive increase above this is due to non-creatinine substances, a rough estimate of the true concentration of creatinine might be derived by extrapolation of the slowly rising curve back to zero time. Estimates derived in this way were practically identical with the results obtained when Fuller's Earth was used in the illustrated examples and on numerous other occasions. The error incurred when Fuller's Earth is not used may be as great as 100 p.c. when dealing with normal children, and is usually about 15 p.c. for the creatinine levels found in normal adults. The error is relatively less when the true creatinine concentration is high.

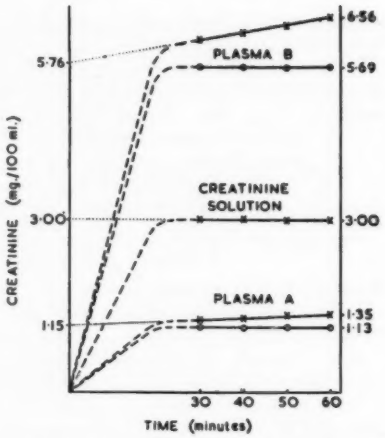

Fig. 1. Development of colour with time, using two methods to measure the true creatinine levels in the plasma of (a) a normal male, and (b) a patient with chronic renal failure: x—x—x old method, used here to calculate true creatinine content by extrapolation of the slowly rising portion of the curve back to zero time; o—o—o Fuller's Earth method as described in this article.

Other evidence comes from a study of the thoroughness with which creatinine added to plasma can be recovered by using Fuller's Earth. Using 5 or 6 p.c. sodium tungstate as the protein precipitant approximately 100 p.c. recovery was effected from normal plasma (Table 1).

Unlike plasma, diluted urine behaved like a simple solution of creatinine. Fuller's Earth offered no advantage: results agreed within 2 p.c. with measurements made by the simpler, straightforward method, and the recovery of added creatinine was 100 p.c. with a range of ± 4 p.c. Heavy proteinuria, blood and a content of 2 p.c. glucose caused no significant interference. Heavy glycosuria with ketonuria was not tested, but may necessitate the use of Fuller's Earth (Owen, 1958); similarly with aminoaciduria and certain drugs (Taussky, 1956).

Protein precipitants. The ideal precipitant would completely remove all the protein from plasma or serum, leave the creatinine unchanged, and cause no disturbance to the colour reaction. Protein which is incompletely removed shows as a faint turbidity in the plasma or serum filtrate; is adsorbed together with creatinine on to Fuller's Earth and then acts as a false chromogen. As there are normally 7,000 mg. of protein to every 1 mg. of creatinine in plasma it is obviously important to have a water-clear filtrate. Somogyi's zinc sulphate

and alkali method is unsatisfactory because creatinine is lost during precipitation.

Folin and Wu's method is satisfactory if modified to give an acid filtrate by using 6 p.c. sodium tungstate. The concentration of reagent is important. When lower concentrations were used the filtrate often showed turbidity which had to be cleared by adding more tungstate, if falsely high readings were to be avoided. When higher concentrations were used, for example, 10 p.c., then the final readings were too low, presumably due to loss of creatinine either by precipitation or by conversion to creatine, and the recovery of added creatinine fell to 93 p.c.

Clearly, the protein content of the test solution should determine the optimal concentration of the precipitant. The use of 6 p.c. sodium tungstate is satisfactory for plasma with a normal content of protein — as shown by the 100 p.c.

TABLE 1.

Recovery of pure creatinine (1 mg./100 ml.) added to plasma samples with normal total protein content, the mean of duplicate estimations being recorded.

Total plasma protein (gm. p.c.)	Plasma creatinine (mg./100 ml.)	Plasma plus 1 mg. p.c. added creatinine (mg./100 ml.)	Recovery (p.c.)
6·0	0·83	1·85	102
6·0	0·75	1·80	105
	0·75	1·76	101
7·0	1·21	2·25	104
6·4	1·17	2·16	99
6·0	1·39	2·43	104
7·0	1·51	2·50	99
6·7	0·84	1·80	96
	Mean of 8 recoveries		101 p.c.

TABLE 2.

Effect of hypoproteinaemia on the measurement of plasma creatinine content using equal volumes of plasma, water, 6 p.c. sodium tungstate, and 0·67N. sulphuric acid for precipitation of protein. The means of duplicate estimations are recorded.

Total plasma protein (gm. p.c.)	Plasma creatinine (mg./100 ml.)	Plasma plus 1 mg. p.c. added creatinine (mg./100 ml.)	Recovery (p.c.)
5·8	0·90	1·88	98
5·7	0·77	1·69	92
5·7	0·92	1·86	94
5·7	0·63	1·54	91
5·6	3·18	4·14	96
5·6	1·04	1·99	95
5·1	2·98	3·95	97
5·1	1·05	1·94	89
4·3	0·73	1·60	87
3·9	0·82	1·70	88
3·5	0·67	1·50	83
3·3	0·78	1·67	89
3·3	0·79	1·63	84

recoveries in Table 1. However, the same reagent may prove to be excessive when added to hypoproteinaemic plasma and recovery of added creatinine may fall to 80 p.c. or less, as shown by the results in Table 2. To cope with this likely source of error it is recommended that creatinine be added to an extra sample of plasma (e.g. 5 ml. of the working standard solution, in place of distilled water, to 5 ml. of plasma) whenever an abnormal concentration of protein is suspected, and that the true plasma creatinine concentration be calculated from the recovery of added creatinine. Alternatively, if the total plasma protein content is known, Lauson's Table can be consulted for an indication of the appropriate amount of protein precipitants to be added (Table 3).

TABLE 3.

Method of precipitation of proteins in hypoproteinaemic plasmas, modified after Lauson.

Total plasma protein (gm. p.c.)	Volumes (ml.)			
	Plasma	Distilled water	6 p.c. sodium tungstate	0·67N. sulphuric acid
5·8-8·0	5	5	5	5
4·5-5·8	5	7	4	4
3·0-4·5	5	9	3	3

Colour reagents. In the original method described by Folin and Wu (1919) the alkaline picrate solution was prepared by mixing five volumes of saturated picric acid and one volume of 10 p.c. sodium hydroxide. The colour reaction was then developed by adding one volume of this mixture to two volumes of the test solution.

We have recommended using different proportions and concentrations of these for three reasons. Firstly, so that the final concentrations of picric acid and sodium hydroxide are the same in both the urine and plasma methods. Thus one volume of saturated picric acid is mixed with one volume of 3 p.c. sodium hydroxide for each method and then made up to ten volumes, with diluted urine in one instance, and with water in the other where Fuller's Earth is to be used. The method thus makes for uniformity, with less pipetting. Secondly, the greater dilution of colour reagents in our method leads to increased sensitivity by lowering the reagent blank. This is illustrated in Table 4.

Thirdly, by adding 5 ml. of colour reagent instead of the usual 7·5 ml., to elute the adsorbed creatinine from Fuller's Earth, optical densities were correspondingly increased in the final solution. Further increase in sensitivity could be achieved, if necessary, by adding still less of the reagent.

Temperature. Colour development is reversibly affected by temperature as illustrated in Fig. 2. The optical density of a mixture removed from a warm water bath decreases gradually as it cools till it assumes a plateau level which is the same as that achieved by mixtures whose colour has been developed en-

TABLE 4.

Effect on the Jaffé reaction of decreasing the concentration of picric acid and sodium hydroxide.

		Folin and Wu method	Present method
Final concentration	Saturated picric acid	28 p.c.	10 p.c.
	Sodium hydroxide (W/V)	0·6 p.c.	0·3 p.c.
Optical density	Reagent blank	1·044	0·201
	Creatinine solution (1 mg./100 ml.)	1·731	1·101
	Difference	0·687	0·900

tirely at room temperature. Rewarming restores the lost colour. The plateau of colour is higher and is reached faster at warm temperatures than at cooler temperatures.

Obviously care has to be taken to measure the colour of the blank and the standard and the unknown solutions at the same temperature; and if creatinine clearance is to be estimated then both plasma and urine samples must be read

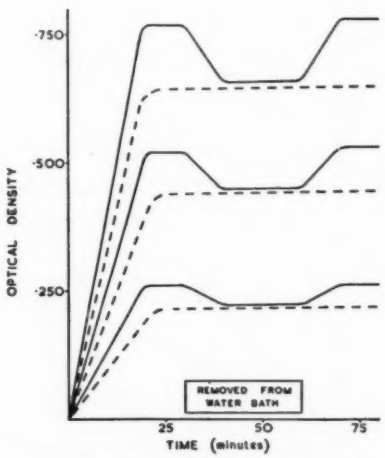

Fig. 2. Reversible effect of change in temperature on colour production. Duplicate samples of three different strengths of creatinine solutions: (a) continuous line for samples treated in water bath at 25° C., then temporarily cooled in air at 17·5° C., (b) broken line for samples kept continuously in air at 17·5° C.

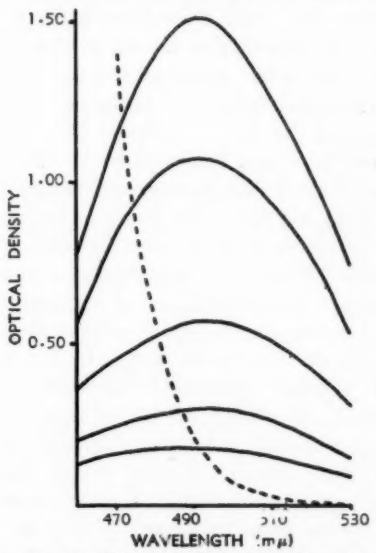

Fig. 3. The optical density of creatinine solutions of five different concentrations (expressed as O.D. of creatinine solution minus O.D. of reagent blank, using distilled water as reference) read at different wavelengths. The dotted line represents the O.D. of the reagent blank.

at the same temperature. Heating is liable to occur during centrifugation and also when solutions are allowed to remain in the spectrophotometer, especially if repeatedly exposed to the light beam. Hence the advantage of measuring the colour of all solutions against distilled water. Accurate measurements of creatinine can be made by reading all solutions at room temperature, but we have recommended always using a water bath at constant temperature so that the actual readings can be checked for comparability throughout the year. This would be unnecessary in a laboratory maintained at a constant temperature.

Thus, two of the most important practical points in measuring creatinine are related to the effect of temperature — (a) that readings should be made immediately after placing the solutions in the spectrophotometer, before alteration in temperature can occur, and (b) that the reference solution should be distilled water rather than the reagent blank, the colour of which varies with temperature.

Spectrophotometry. Some workers recommend measuring optical density at a wavelength selected so as to give a low reading for the reagent blank, for example, 520-550 mμ (Bonsnes and Taussky, 1945; Owen *et al.*, 1954). This apparent advantage is offset by two drawbacks. First, even though the O.D. of the reagent blank is lower at 520 mμ than at 490 mμ, the difference in optical densities of a creatinine solution and the reagent blank is maximal at 490 mμ: thus, sensitivity is greatest at the optimal wavelength recommended here (Fig. 3). Secondly, Beer's Law is obeyed over a wide range of concentrations (Fig. 4) only when the optimal wavelength is used.

A quartz-prism spectrophotometer such as the Beckman Model DU used here has been found to be the most satisfactory instrument. Photometers employing filters are liable to be less satisfactory because of the departure from Beer's Law which occurs with higher concentrations of creatinine when the wavelength is not precisely optimal. This is illustrated in Fig. 4. A popular colorimeter such as the E.E.L. is satisfactory for routine use, provided the unknown solutions are diluted initially, before colour development, to bring the readings onto the more linear part of the calibration curve. Instruments using a diffraction grating are satisfactory within the limits of their printed scale.

The Beckman Model DU quartz-prism spectrophotometer gives a

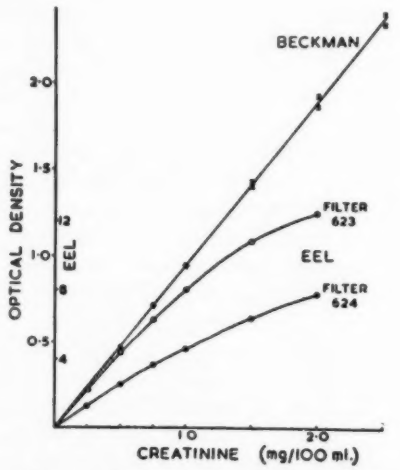

Fig. 4. Optical density of standard solutions of creatinine showing the straight line achieved by the Beckman spectrophotometer using a wavelength of 490 mμ. As comparison, the curves obtained with the E.E.L. colorimeter are shown, using filters 623 and 624.

linear relationship between optical density and creatinine concentration up to a plasma level of 10 mg./100 ml. with the 1 in 4 dilution described under methods (Fig. 4). The Bausch and Lomb "Spectronic 20" which uses a diffraction grating gives a satisfactory linear calibration curve at 490 mμ, but only up to a plasma level of 2 mg./100 ml. The E.E.L. photoelectric colorimeter using coloured filter No. 623 gives a linear calibration only up to 1·6 mg./100 ml.: further dilutions would increase the range but decrease accuracy.

Standard solutions. It is important to be aware of the readiness with which creatinine in solution is liable to be converted to creatine.

The recommended stock standard of 1 mg. of creatinine in 1 ml. of 0·1N hydrochloric acid remains stable for many months. The creatinine zinc chloride equivalent, 1·61 mg. in 1 ml. of 0·1N hydrochloric acid, is also satisfactory. A neutral solution of creatinine in water, however, shows a gradually decreasing concentration of creatinine and a rising content of creatine. When a 5 p.c. solution of creatinine was put into ampoules, 100 p.c. of the nitrogenous contents was initially creatinine; after nine months, 72 p.c. was creatinine and 28 p.c. creatine; and after eighteen months, 52 p.c. was creatinine and 48 p.c. creatine. The total nitrogen content remained constant. Hydrolysis of creatinine proceeds more rapidly in dilute solution. For example, 25 p.c. of the creatinine in a 0·001 p.c. solution became converted to creatine in two weeks. The stability of stock solutions can be checked by measuring their creatinine content before and after conversion of any creatine to creatinine by boiling 1 ml. of solution with 4 ml. of N hydrochloric acid for three hours. The true creatinine content of solutions should be measured by the Fuller's Earth method to avoid interference by creatine.

Degree of precision of the method. The standard deviation can be calculated for duplicate readings on different samples of plasma and urine by using the formula $\sqrt{\frac{\Sigma d^2}{2n}}$, where d = the difference between duplicates in mg. of creatinine per 100 ml., and n = the number of samples tested. The results are given in Table 5.

TABLE 5.

Precision of the method as calculated from duplicate measurements on different samples of plasma and urine.

Creatinine solutions	Number of solutions	Mean value (mg./100 ml.)	Standard deviation	
			mg./100 ml.	percentage of the mean
Plasma	30	2·46	0·040	1·6 p.c.
Urine	30	67·4	0·71	1·1 p.c.

DISCUSSION.

These results agree with those of other workers in suggesting that creatinine can be measured with considerable accuracy in both plasma and urine (Hare, 1950; Owen *et al.*, 1954). Plasma demands a more complex method than urine but both methods have several important requirements in common. One is rigid temperature control, as the colour reaction varies with temperature; an additional safeguard in this connection is to make all the final colorimetric measurements against water and not against reagent blank whose temperature, and colour, may alter with repeated use. Secondly, the colorimetric reading of the reagent blank should be kept low. To do this by appropriately diluting the colour reagent, as described here, seems preferable to the unsatisfactory method of measuring optical densities at a wavelength other than the optimum. Thirdly, calibration must be carried out with proper standard solutions of creatinine, because of the liability of conversion to creatine. And finally, due regard must be paid to the characteristics of the apparatus being used to measure the intensity of the colour, especially when the simpler machines having filters or a diffraction grating are employed.

In dealing with plasma or serum, additional factors of great importance relate to the use of Fuller's Earth and protein precipitants.

Fuller's Earth. Fuller's Earth, or Lloyd's Reagent, is used on the assumption that it effects a complete separation of creatinine from all other interfering chromogens by adsorbing creatinine in an acid medium and releasing it in an alkaline medium. Evidence in support of complete adsorption of creatinine from watery solutions, from urine and from plasma freed of protein comes from our own work and that of various other workers.

(1) If Fuller's Earth is used in an acid solution of pure creatinine, all the creatinine is recovered quantitatively from the button of Fuller's Earth, and no chromogen remains in the supernatant fluid.

(2) If creatinine is added to plasma filtrates or diluted urine, 100 p.c. recovery is obtained after adsorption on Fuller's Earth.

(3) If creatinine is added to plasma before protein precipitation, 100 p.c. recovery is obtained if the correct proportions of protein precipitants have been used.

(4) Estimates of creatinine in plasma obtained by using Fuller's Earth agree with estimates derived by extrapolating to zero time the slowly rising curve of colour density produced in the older method. This was discussed earlier and illustrated in Fig. 1, and assumes that the rising colour above a plateau is due to non-creatinine chromogens.

(5) Plasma creatinine levels estimated by using Fuller's Earth approximate to those obtained by the specific method using NC-bacteria (Miller and Miller, 1951; Owen *et al.*, 1954).

Evidence that Fuller's Earth adsorbs only creatinine and none of the other Jaffé-positive chromogens present in protein-free filtrate is as follows:

(i) Colour development in the eluate from Fuller's Earth is identical with that of a pure solution of creatinine, provided the filtrate is protein-free.

(ii) Paper chromatography of the eluate from Fuller's Earth shows only one Jaffé-positive spot which is identical with that of pure creatinine. This is in contrast to an ultrafiltrate or acetone extract of plasma, not treated with Fuller's Earth, which produces two distinct Jaffé-positive spots and streaking (Ralston, 1955).

(iii) Fuller's Earth does not adsorb any of the chromogen remaining after filtrates have been treated with NC-bacteria, which destroy creatinine completely (Miller and Miller, 1951; Owen *et al.*, 1954).

(iv) Fuller's Earth does not adsorb any of the chromogen remaining after dialysing plasma against running tap water for six days, a procedure which removes endogenous creatinine and added creatinine completely (Owen *et al.*, 1954).

Protein precipitation. When Fuller's Earth is used, and all other precautions are taken, then the factor most likely to affect the accuracy with which creatinine is measured is protein precipitation. In common with others (Lauson; Haugen, 1953; Owen *et al.*, 1954) we have found that sodium tungstate and sulphuric acid in the concentrations used here rather than in the less acid form originally described (Folin and Wu, 1919), are completely satisfactory for normal plasma. When the concentration of protein in plasma is less than normal, however, falsely low estimates of creatinine are obtained, and the recovery of added creatinine is incomplete and is possibly related to increase in pH of the filtrate above 2. This has also been noted by Lauson. The error can be corrected by one or both of the following methods.

Smaller amounts of protein precipitants can be added in direct proportion to the total protein concentration according to the scheme suggested by Lauson (Table 3).

The method we have used is to estimate the recovery of creatinine added to a separate portion of the patient's plasma, and to correct the original determination accordingly.

Lack of recognition of this error associated with hypoproteinaemia would lead to underestimation of the plasma concentration of creatinine and overestimation of the renal clearance of creatinine. It can occur with any of the methods used for measuring plasma creatinine and especially where 10 p.c. tungstate has been used as the protein precipitant. It is of considerable importance when estimations of creatinine clearance are being used to follow progress in diseases such as nephrosis and probably invalidates many of the published figures (Mattar *et al.*, 1952).

We believe it is possible to measure creatinine accurately and that many of the criticisms levelled against the usefulness of creatinine estimations in

the past are invalid because they were founded on erroneous measurements. Claims that creatinine clearance did not agree with inulin clearance, for example, were based on measurements of the total chromogen content of plasma and not on the true creatinine content (Smith, 1951). By contrast, Haugen and Blegen (1953) and Owen, Robson, *et al.* (1955) found good agreement between the two clearances, using Fuller's Earth for measuring true creatinine. If this is true, the measurement of plasma creatinine and endogenous creatinine clearance would be as useful as the more complex inulin clearance and would be more informative and more convenient than the corresponding measurements of urea which are almost universally made in clinical practice.

SUMMARY.

Methods are described for measuring the true creatinine, as opposed to the total chromogen, concentration in plasma and urine. Tungstic acid is used as a protein precipitant and Fuller's Earth as an adsorbent of creatinine in plasma. A simpler method for urine is satisfactory. Important aspects of protein precipitation, temperature and colorimetry are stressed. Attention is drawn to the error likely to occur when dealing with hypoproteinaemic plasma and a method of correction is described.

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TYPE SPECIFIC ANTIBODIES TO *STREPTOCOCCUS PYOGENES*

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Many attempts have been made to measure type-specific antibody to *Streptococcus pyogenes* in human sera, but none has proved entirely satisfactory. The bactericidal test, useful for determining the type specificity of streptococcal strains and antisera (Maxted, 1956; Lancefield, 1957), has been used by several workers, including Rothbard (1945) and Denny, Perry and Wannamaker (1957), to examine the antibody content of small numbers of human sera. However, because it is necessary to use living human leucocytes in blood which is free from antibody to the strains being tested, the technique is too cumbersome for the examination of large numbers of sera with a wide range of antigenic types.

Boyden (1951) demonstrated that a protein antigen of *Str. pyogenes* could be adsorbed to sheep red cells which had been treated with tannic acid. This technique was employed by Denny and Thomas (1953) who used a haemagglutination test to titrate type specific antibodies, but because of the frequency of non-type specific cross reactions they were doubtful of the value of their results. Denny and Thomas found that the use of a purified M antigen obtained from Dr. R. C. Lancefield did not improve the specificity of their test and they also attempted, but with only partial success, to neutralize the cross reacting material with heterologous antigens.

The experiments described in this paper were designed to improve the specificity of the haemagglutination test by suitable absorption of sera prior to testing.

Materials and Methods.

Bacterial cultures. Stock type strains of *Str. pyogenes* were obtained from the Streptococcal Reference Laboratory, Colindale. These, and locally isolated strains of *Str. pyogenes*, were maintained in the dried state. The stock strains were as follows:

SF130/13 (type 1), Richards GIII (type 3), R51.776 (type 5), S43/78 (type 6), 3722/17 (type 12), Lowe (type 14), J17E/19 (type 17), J17C (type 18), J17D/50 (type 19), T23/Rb5/26 (type 23), C98/97 (type 24), J17F/51 (type 26), D23/11 (type 29), D24/46 (type 30), 44273 (type 31), C107/24/5 (type 33), C119/58/1 (type 36), C105 (type 46), C744/Rb4/41 (type 47).

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Rabbit antisera were prepared by immunization with heat-killed or formalin-killed vaccines. Sera were preserved with 1 in 10,000 merthiolate and stored at 4° C.

Human sera were stored, without preservative, at minus 15° C. in a deep freeze.

Culture media. Heat sterilized Todd-Hewitt broth, containing neopeptone (Difco) and phenol red, was used for preparing Lancefield extracts and absorbing suspension. An 800 ml. volume of broth was inoculated with 5 ml. of an actively growing culture. After 16 hours' incubation at 37° C. 30 ml. of 50 p.c. glucose were added and the culture was neutralized with 40 p.c. NaOH intermittently during a further 8-hour period of incubation.

Lancefield extracts. The deposit from an 800 ml. culture was washed once in saline, extracted with 10 ml. of N/5 HCl for 10 minutes in a boiling water bath and neutralized with N/2 NaOH until added phenol red just became pink. The cells were re-extracted first with 8 ml. and then with 6 ml. of N/5 HCl. The three extracts were pooled and the presence of M antigen was confirmed by the precipitin test (Swift, Wilson and Lancefield, 1943).

Absorbing suspension. The centrifuged deposits from 800 ml. cultures were washed once in saline, suspended in a small volume of saline and heated at 56° C. for 30 minutes. This suspension was stored at 4° C. and used within 4 weeks of preparation.

Saline solutions were freshly prepared every few days and used without autoclaving since it was found that red cells became very sensitive to tannic acid if washed and suspended in saline which had been autoclaved in ordinary glassware.

Saline—8·5 gm. NaCl in 1000 ml. of distilled water.

Saline buffer (pH 7·2)—4·25 gm. NaCl, 8·1 gm. Na_2HPO_4, 3 gm. KH_2PO_4 in 1000 ml. of distilled water.

Rabbit serum saline buffer (RSSB)—saline buffer with either 0·04 p.c. or 1 p.c. normal rabbit serum added.

Sheep red blood cells were collected by jugular puncture into Alsever's solution and stored at 4° C. They were suitable for use for at least 4 weeks.

Tannic acid treatment. Red cells were washed three times with saline and a 2 p.c. suspension made up in saline buffer. An equal volume of 1/25,000 tannic acid in saline, prepared on the morning of the test, was added and mixed. After standing for 10 minutes at room temperature the cells were washed once in, and made up to 2 p.c. in saline buffer.

Sensitized cells. One volume of 2 p.c. tanned cells was mixed with one volume of antigen diluted in saline buffer and allowed to stand for 30 minutes at room temperature. The cells were lightly centrifuged, washed once in 0·04 p.c. RSSB and resuspended to 4 p.c. in the same medium.

Preparation of sera. Freshly collected sera were heated at 56° C. for 20 minutes but stored sera did not require inactivation. Details of the absorption of sera with whole streptococci will be given in the text. After this absorption sera were treated with 2 volumes of packed sheep cells for 1 hour at room temperature to remove haemagglutinins.

The test proper. Using dropping pipettes, graduated to deliver 0·02 ml. and transparent plastic trays, 5-fold serial dilutions of serum (0·16 ml.) were made in 1 p.c. RSSB. Two drops (0·04 ml.) of sensitized cell suspension were then added to each dilution and mixed by agitation of the tray which was then incubated for 30 minutes at 37° C. Cells were then resuspended by gentle shaking and allowed to settle at room temperature. Since there was little difference between a 2-hour reading and an overnight reading it was decided to record results as soon as the negative control tests had finally settled. The reciprocal of the last serum dilution which produced definite agglutination was regarded as the titre of the serum.

Controls were included in each test to show that the sensitized cells were not agglutinated by normal rabbit serum and that the antisera under test did not agglutinate unsensitized tanned cells.

EXPERIMENTAL.

Preparation of antigen. Lancefield extracts were prepared from stock strains of those types of *Str. pyogenes* which readily produced rabbit M antisera and also from selected recently isolated strains.

Successive volumes of absolute alcohol were added to each Lancefield extract and the precipitates from the ½, 1, 2, 3 and 4 volume fractions were collected. Each precipitate was dissolved in saline to one-third of the original volume of the Lancefield extract, heated for 10 minutes in a boiling water bath to drive off the alcohol, and stored at 4° C. with 1/10,000 merthiolate as preservative.

Each fraction was used to sensitize tanned cells which were then tested against absorbed homologous serum and against a single unabsorbed heterologous serum. Results varied with different preparations, but in general heterologous reactions were strongest in the ½, 1 and 4 volume fractions, whereas homologous reactions were strongest in the ½, 1 and 2 volume fractions or sometimes equally strong in all fractions. The 2 volume fraction has proved the most specific for all extracts tested and is subsequently referred to as the antigen.

Test dilution of antigen. One drop (0·02 ml.) of the chosen dilution of antigen was added to 1 ml. of saline-buffer and used to sensitize 1 ml. of 2 p.c. tanned cells. Antigens were used at dilution between 1/2 and 1/32. Dilution was necessary either because the neat extract caused spontaneous agglutination of tanned cells, because cross reactions could be avoided, or because dilution of the antigen did not reduce the titre of the homologous serum. Antigens have kept satisfactorily for more than three months.

Absorption of sera. When the test dilution of each antigen was tested against a 1/25 dilution of all the type antisera numerous non-specific reactions were found. These stock precipitin sera had been absorbed with type 11 (T11/54/4) or strain Hempston and showed no cross reactions in routine precipitin tests. Further absorption with these stock absorbing suspensions failed to remove the cross reactions.

Since the type 14 serum showed numerous cross reactions the type 14 strain (Lowe) was tested to see whether it could absorb out the non-specific reactions. Crude type 5, type 6 and type 14 sera were absorbed at a dilution of 1/25 with two volumes of packed type 14 cells. The results in Table 1 show that non-specific reactions were removed by this absorption.

All the crude sera were then absorbed with two volumes of type 14 (Lowe) suspension and tested against each type antigen. Non-specific cross-reactions between types 5, 6, 14, 18, 19, 30, 46 and 47 had been removed, but other cross-reactions remained. Absorption with type 31 (44273) was successful in removing the remaining most troublesome cross-reaction between types 3, 12, 18 and 31 but was not able to replace type 14 absorption. For this reason a combined absorption with types 14 and 31 was tested.

TABLE I.

Comparison of titres of rabbit sera before and after absorption.

Antiserum		Titre of serum against antigen of type:		
Type	Absorbed with	5	6	14
5	Not absorbed	15,625	25	125
6	Not absorbed	125	3,125	125
14	Not absorbed	625	625	3,125
5	Type 14	15,625	—	—
6	Type 14	—	3,125	—
14	Type 14	—	—	—

Table 2 shows the reactions of the sera after simultaneous absorption with two volumes of type 14 and two volumes of type 31 cells. Despite this heavy absorption homologous titres were high and most antigens reacted only with homologous sera. Several minor cross-reactions remained, there was a reciprocal cross between type 18 and type 36, and the type 47 antigen was non-specific.

Nature of the sensitizing antigen. Some of the characteristics of the antigen were examined in the following experiments.

Samples of a type 12 rabbit antiserum (R.67) were each absorbed with a single volume of three different absorbing suspensions. The first sample was absorbed with untreated homologous type 12 suspension prepared from a strain containing M antigen, the second with a portion of the same suspension which had been treated with trypsin for 16 hours at 37° C. to destroy the M antigen and the third with a suspension of a locally isolated strain (3611) which was agglutinated by type 12 T antiserum, but which did not precipitate with type 12 M antiserum. The absorbed sera were then titrated against cells sensitized with type 12 antigen. The titre of the unabsorbed serum was 31,250 and of the three absorbed samples, < 10, 31,250 and 6,250 respectively. The untreated type 12 suspension removed all the specific antibody from the serum but the other two suspensions failed to reduce the titre appreciably.

The behaviour of sera in the haemagglutination test was compared with reactions in the precipitin test. Sera from rabbits immunized with three different type 12 strains were tested against all three strains in the precipitin test and against two strains in the haemagglutination test. The results are shown in Table 3. Serum R67/4 which reacted strongly in the precipitin test had the highest haemagglutination titre, but sera which gave weak or negative precipitin reactions also had quite high titres of haemagglutinating antibody.

The three type 12 sera (R67, R127 and R139) were then tested with all antigens before absorption, after absorption with type 14 and after a further absorption with type 31. There were differences in the cross-reaction pattern of unabsorbed sera but after absorption with type 14 all three sera reacted only with antigens of types 3, 12 and 31 and the final type 31 absorption rendered all sera specific for type 12 antigen.

TABLE 2.

Titres of rabbit antisera after combined absorption with suspensions of types 14 *and* 31.

Anti-serum of type:—	Cells sensitized with antigen of type:—																		
	1	3	5	6	12	14	17	18	19	23	24	26	29	30	31	33	36	46	47
1	3,125	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	—	3,125	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	15,625	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	—	—	—	3,125	—	—	—	—	—	—	25	—	—	—	—	—	—	—	—
12	—	—	—	—	3,125	—	—	—	—	—	—	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
17	—	—	—	—	—	—	625	—	—	—	—	—	—	—	—	—	—	—	—
18	—	—	—	—	—	—	—	78,125	—	—	—	—	—	—	—	—	15,625	—	—
19	—	—	—	—	—	—	—	—	3,125	—	—	—	—	—	—	—	—	—	—
23	—	—	—	—	—	—	—	—	—	78,125	—	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	—	—	—	—	3,125	—	—	—	—	—	—	—	25
26	—	—	—	—	—	—	—	—	—	—	—	3,125	—	—	—	—	—	—	625
29	—	—	—	—	—	—	—	—	—	—	—	—	625	—	—	—	—	—	—
30	—	—	—	—	—	—	—	—	—	—	—	—	—	625	—	—	—	—	—
31	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
33	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	390,625*	—	—	—
36	—	—	—	25	—	—	—	125	—	—	—	—	—	—	—	—	625	—	—
46	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	15,625	—
47	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3,125

— = less than 25. * The type 33 serum had a prozone (1/25 was negative, 1/125+, 1/625+++).

TABLE 3.

Comparison of the precipitin reaction and haemagglutinin titre of three type 12 antisera.

Rabbit No.	Immunizing strain	Precipitin reaction*	Haemagglutinin titre against antigen	
			3722/17	Ts.1005
R67/4	3722/17	***	15,625	78,125
R127/2	S54/2365	—	3,125	3,125
R139/3	Ts.1005	trace	625	3,125

* Identical results were obtained with extracts prepared from the three immunizing strains.

Further evidence that type-reactions were type-specific, rather than strain specific, was obtained when antigens from selected locally isolated strains (types 1, 3, 5, 6, 12 and 19) were tested against rabbit typing sera prepared against stock strains. Many stock strains had been mouse-passaged repeatedly. Antigens from non-mouse-passaged locally isolated strains all reacted satisfactorily with homologous type sera and some proved more potent than antigens prepared from stock strains.

Type specific haemagglutinating antibody was present in antisera prepared against both heat- and formalin-killed vaccines. Non-type specific antibody was also found in both kinds of antisera. Of the two sera which showed the greatest number of cross-reactions one (type 14) had been prepared against a formalin-killed vaccine, and the other (type 18) against a heat-killed vaccine.

Haemagglutination tests with human sera. Since reactions with rabbit antisera, after appropriate absorption, appeared to be both sensitive and type-specific tests were carried out with 52 human sera which had been collected from patients, mostly children, at the time of tonsillectomy.

Human sera were absorbed in the same manner as rabbit sera. A 1 in 25 serum dilution was absorbed with two volumes of type 14 together with two volumes of type 31 cells. A second absorption with two volumes of packed sheep cells followed. Absorbed sera were then tested with all prepared type antigens, excluding type 14 and type 31. When positive reactions were observed sera were retested at higher dilutions.

One human serum still agglutinated the tanned cell control in spite of the previous sheep cell absorption, a difficulty occasionally encountered with rabbit sera, but the remaining 51 sera reacted satisfactorily. Twenty of the 51 sera failed to react with any of the antigens. Of the 31 sera which did react, 16 agglutinated with one antigen, 7 with two, 5 with three, and 3 with four antigens.

As shown in Table 4 agglutination was observed with only 8 of the 17 antigens employed in the test.

TABLE 4.

Result of testing 51 human sera with 17 type antigens.

Antigen of type:	Number of sera reacting
1	11
3	6
5	3
6	13
12	18
18	3
30	1
36	2
17, 19, 23, 24, 26, 29, 33, 46, 47.	0

It will be seen that reactions with type 12, type 6 and type 1 were obtained much more frequently than reactions with other types. This distribution accords well with the frequency of *Str. pyogenes* types isolated in this hospital from streptococcal infections and from carriers over the 6 months prior to collection of the sera. Three types were predominant amongst the 389 strains of *Str. pyogenes* isolated–type 6 (58 strains), type 1 (50 strains) and type 12 (34 strains).

Of the three sera which reacted with type 18 antigen two also reacted with type 36 antigen. Since rabbit sera showed a cross relationship between these two types it is probable that antibody to only one of them was present. There was no noticeable correlation between reactions to any other types and single reactions to each of types 1, 3, 6 and 12 occurred in individual sera. Titres of 125 were observed fifteen times and of 625 five times.

Sera which react with several antigens might do so either because they contain antibodies to several types or because an antigen shared by several types had not been removed by absorption. The following experiment shows that, in the example given, as in other sera which have been tested, multiple reactions indicated the presence of multiple type antibodies.

Samples of a human serum which reacted after basic absorption with antigens of three types (types 1, 12 and 19) were absorbed separately with types

TABLE 5.

Multiple antibodies in a human serum.

Treatment of serum	Antibody titre to		
	type 1	type 12	type 19
Before specific absorption	625	625	625
After absorption with type 1	—	125	625
After absorption with type 12	625	—	625
After absorption with type 19	625	625	—

— = less than 5.

1, 12 and 19 cells and retested against each of the three antigens. The results are shown in Table 5. Absorption with each of types 1, 12 and 19 abolished the homologous reaction but did not alter the titre against the other two types by more than one dilution.

DISCUSSION.

These results show that by using a heavy absorption with two particular strains of streptococci it is possible to obtain type specific rabbit sera for use in the haemagglutination test. Sera satisfactorily absorbed for precipitin tests still show too many cross-reactions for haemagglutination tests. Type specific antibody can withstand heavy heterologous absorption but, in those types tested, could readily be removed by specific absorption.

The results with human sera are theoretically more difficult to interpret because multiple specific antibodies are likely to be present and there might be additional non-specific antibodies produced during infection with live organisms. However, it does appear that the absorption technique used here will render human sera as specific as rabbit antisera and that multiple reactions do indicate multiple antibodies.

The sensitizing antigens used were trypsin-sensitive, heat-stable proteins which were precipitated by two volumes of alcohol, but whether they are indeed M antigens is uncertain. Sensitizing antigen is obtainable from strains of all types of *Str. pyogenes* which readily produce M antisera in rabbits but its presence in strains of those types which fail to produce good rabbit precipitin sera has not been adequately investigated. The results in Table 3 show that haemagglutinating antibody could be demonstrated in antisera which were not strong enough to react in the precipitin test.

Because T antigens are heat-sensitive, and because the cross-reactions of unabsorbed sera do not resemble known T antigen crosses, it seems unlikely that T antigens play an important rôle in haemagglutination reactions. It is possible, although the cross-reaction patterns are not identical, that the non-specific antibodies shown by the haemagglutination technique may be related to the non-type-specific mouse protective antibodies described by Alice Evans (1946) and by Fleck (1956).

It does appear that the haemagglutination test can be modified, by suitable absorption of the sera, to yield results which indicate the presence of type specific antibody in both rabbit and human sera. Moreover, this technique should prove useful in epidemiological work because large numbers of sera can readily be tested with a wide range of serological types of *Str. pyogenes*.

SUMMARY.

Non-specific reactions in a haemagglutination test can be virtually eliminated from rabbit antisera by heavy absorption with certain strains of whole streptococci.

Antigens prepared from 19 types of *Str. pyogenes*, comprising those types which give rise to good precipitin sera, reacted with their homologous rabbit sera after absorption.

Preliminary tests suggest that type specific reactions may be obtained and multiple antibodies may be demonstrated in human sera.

The nature of the sensitizing antigen has not been determined.

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